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MUDr. Jan Bouček

Imunologicko-biologické aspekty spinocelulárních karcinomů v oblasti hlavy a krku

Immuno-biological Aspects of Head and Neck Squamous Cell Carcinoma

Disertační práce

Školitelé:

Prof. RNDr. Blanka Říhová, DrSc.

Prof. MUDr. Jan Betka, DrSc.

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MUDr. JAN BOUČEK

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Abstrakt:

Vznik nádorového onemocnění je podmíněn změnou či sérií změn na genové úrovni. Rozvoj nádorového onemocnění je do značné míry touto změnou předznamenán, ale velmi významnou roli zde hrají další okolnosti. V případě solidních nádorů se jedná hlavně o nádorové mikroprostředí. Zde se setkávají samotné nádorové buňky s buňkami okolního stromatu, zejména fibroblasty, a s buňkami imunitního systému. Zde se také zásadním způsobem formuje charakter a intenzita protinádorové imunitní odpovědi.

Spinocelulární karcinom hlavy a krku je 6. nejčastější onkologické onemocnění postihující každoročně celosvětově více než půl milionu pacientů. Přes pokroky a zlepšení ve všech léčebných modalitách nedosahuje terapie ani v západních zemích uspokojivých hodnot a již několik desetiletí setrvávají na přibližně stejných hodnotách. V současné době je pravděpodobnost přežití 5 let, bez ohledu na lokalizaci a stádium onemocnění, jen přibližně 40%.

V předložené práci jsou diskutovány imunologické a biologické aspekty spinocelulárních karcinomů hlavy a krku shrnující poslední poznatky o molekulární podstatě chování nádorových buněk, o vlivu a významu regulace imunitního systému pro klinický průběh onemocnění, a o moderních terapeutických přístupech.

Klíčová slova: Spinocelulární karcinom hlavy a krku, regulační T lymfocyty, protinádorová imunita, regulace imunitní odpovědi, nádorové mikroprostředí

Abstract:

The process of tumorigenesis is conditioned by change or the series of changes at a gene level. The development of cancer is largely pre-ordained by this change, but very important role is played by other factors. In case of solid tumors it is mainly a tumor microenvironment, where the tumor cells are in contact with stromal cells, especially fibroblasts, and immune cells. Tumor microenvironment can also critically modify the nature and intensity of anti-tumor immune response.

Squamous cell carcinomas of the head and neck are the sixth most common cancer, which affect each year more than half a million patients worldwide. Despite advances and improvements in all treatment modalities, achieved therapeutic results even in Western countries are not satisfactory and remain at approximately the same values for several decades. At prezent, the 5 years survival rate, regardless of the location and stage of disease, is approximately only 40%.

In the prezented work the immunological and biological aspects of squamous cell carcinoma of the head and neck are discussed. It summarizes the recent findings on the molecular basis of the behavior of tumor cells and the influence and significance of regulation of the immune system for the clinical course of disease and the modern therapeutic approaches.

Key words: Head and Neck Squamous Cell Carcinoma, T regulatory cells, cancer immunity, regulation of immune system, cancer microenvironment

Poděkování

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Prof. RNDr. Blance Říhové, DrSc.

(Mikrobiologický ústav AVČR, v.v.i.)

a

Prof. MUDr. Janu Betkovi, DrSc.

(Klinika otorinolaryngologie a chirurgie hlavy a krku 1. LF UK, FN v Motole)

V Praze 23.5.2011

MUDr. Jan Bouček

Obsah

Úvod

Nádorová onemocnění jsou jedním z hlavních celosvětových problémů, který dle statistik Světové zdravotnické organizace (WHO) zapříčinil v roce 2007 smrt asi 7,9 milionu lidí, což činí asi 13% všech umrtí. Počet osob zemřelých v důsledku onkologických onemocnění stále roste a odhaduje se, že se bude i nadále zvyšovat. Předpokládá se až 12 milionů zemřelých v roce 2030. V rozvinutých zemích jsou onkologická onemocnění druhou nejčastější příčinou úmrtí po kardiovaskulárních chorobách. Alarmujícím faktem je, že při optimálním fungování preventivních programů by bylo možné zabránit až 50% těchto úmrtí [1].

75% všech zemřelých na nádorové onemocnění jsou obyvatelé málo a středně rozvinutých zemí. Celosvětově mezi nejčastější patří nádory plic (1,3 mil/rok), žaludku (0,8 mil/rok), tlustého střeva (0,64 mil/rok), jater (0,61 mil/rok) a prsu (0,51 mil/rok). V závislosti na mnoha faktorech, jako je genetická zátěž, nutriční parametry, expozice cigaretovému kouři, alkoholu či slunečnímu záření, výskyt infekčních chorob apod. se incidence v jednotlivých zemích výrazně liší. Zároveň se liší nejčastější typy nádorů u mužů a u žen, kdy u mužů jsou nejčastější příčinou umrtí nádory plic, žaludku, jater, kolorektální, jícnu a prostaty, u žen pak nádory prsu, plic, žaludku, kolorektální a karcinomy děložního čípku [2].

V České republice se novotvary podílely na celkově 28,2% úmrtí u mužů, respektive 26,9% u žen. V roce 2009 zemřelo celkem 54080 mužů, z toho 24051 na onemocnění srdce a oběhové soustavy, 15 673 na novotvary, 3 505 na onemocnění dýchací soustavy, 2 707 na onemocnění trávicí soustavy, 526 na infekční onemocnění, 4 176 na vnější příčiny nemocnosti a úmrtnosti (= poranění a otravy). Statistika ženského pohlaví ve stejném období zahrnuje 58988 celkem zemřelých, z toho 30049 na onemocnění srdce a oběhové soustavy, 12391 na novotvary, 2888 na onemocnění dýchací soustavy, 2099 na onemocnění trávicí soustavy, 596 na infekční onemocnění, 1770 na vnější příčiny nemocnosti a

úmrtnosti (= poranění a otravy). Z pohledu do statistik je patrné, že se postupně snižuje počet zemřelých na onemocnění oběhové soustavy a zvyšuje se podíl zemřelých z důvodu zhoubného novotvaru. Tento trend koresponduje s obdobnou situací v dalších západních zemích [3].

Maligní nádor je definován schopností rychle tvořit množství abnormálních buněk, které se chovají autonomně, svým růstem nerespektují přirozené hranice, mohou se propagovat do okolních částí těla a šířit i do vzdálených tkání či orgánů (proces metastazování). Metastazování je pak hlavní příčinou většiny umrtí na nádorová onemocnění.

Nádorový klon je nejspíše odvozen z jedné buňky či skupiny buněk. V současné době víme, že pro vznik nádoru je zapotřebí kombinace genetické dispozice a působení zevního prostředí. Změna normální buňky na nádorovou je proces probíhající v několika krocích, typicky se odehrávající v premaligní lézi, která se následně změní na maligní proces. Tato změna je důsledkem interakce mezi genetickými faktory a třemi kategoriemi zevních faktorů: 1) fyzikální karcinogeny (UV záření, ionizující záření), 2) chemické karcinogeny (azbest, složky tabákového dýmu, aflatoxiny, arsen), 3) biologické karcinogeny (virové infekce, bakterie, parasité). Účinnost či efekt jednotlivých zevních faktorů však do značné míry závisí na genetické predispozici dané populace či tkáně. Příkladem může být japonská populace žijící na Hawaji, která, přestože nebyla ovlivněna explozí jaderné bomby v Hirošimě a v Nagasaki, má téměř stejně vysoké procento výskytu okultních nádorů štítné žlázy jako populace žijící v Japonsku (24% na Hawaji a 28% v Japonsku), kdy celosvětově se incidence okultních nádorů štítné žlázy (náhodných, bez předchozí klinické manifestace) nalezených v průběhu pitvy pohybuje od 0,1% do 10% (krom jedné práce finských autorů z roku 1985, kteří uvádí 36%) [4-7]. Příčinná souvislost mezi zevním faktorem a onemocněním byla prokázána pro celou řadu nádorů, tyto faktory jsou pak označovány jako rizikové faktory, jedním z nejdůležitějších je kouření. WHO uvádí, že eliminací

rizikových faktorů by bylo možné předejít až 30% úmrtí v důsledku onkologických onemocnění.

Některé příklady nádorů vznikajících v souvislosti s infekcí (ať již na podkladě přímého působení infektu či na podkladě infektem indukovaného chronického zánětu či oslabení imunitního systému): 1) virus hepatitidy B a hepatální karcinom; Human Papilloma Virus (HPV) a nádory čípku děložního a nádory v oblasti orofaryngu; human immunodeficiency virus (HIV) v kombinaci s lidským Herpes virem 8 (HHV8) a Kaposiho sarkom; virus EBV (Epstein-Barrové virus), u kavkazské populace často asociován s klasickým Hodgkinovým lymfomem, s Burkitovým lymfomem v afrických zemích a nasofaryngeálním karcinomem v jihovýchodní Asii, 2) baktérie - Helicobacter pylori a nádory žaludku a lymfomy (MALT), 3) parazité - schistosomósa a nádory močového měchýře.

Dalším důležitým faktorem ovlivňujícím rozvoj nádorů je věk. Incidence dramaticky roste s věkem, zejména díky nahromadění specifických rizikových faktorů. Celkové nahromadění rizik je kombinováno s věkem se snižující efektivností buněčných reparačních mechanizmů. Někdy je tento stav také nazýván věkově závislá imunodeficience (podmíněná zejména sníženou obnovou T buněčných populací v důsledku involuce brzlíku, sníženého množství CD8+ naivních buněk a snížené exprese molekuly CD28) [8].

V málo a středně rozvinutých zemích jsou hlavními rizikovými faktory kouření, konzumace alkoholu, malnutrice, nízký příjem ovoce a zeleniny, chronické infekce jako hepatitida B (HBV) a C a některé typy HPV. V kontrastu s tím jsou v západních zemích hlavními rizikovými faktory krom kouření a konzumace alkoholu hlavně nadváha a obezita.

Existuje celá řada znalostí o příčinách nádorových onemocnění, z čehož vyplývá řada možností jak jim předcházet. Množství onkologických onemocnění lze jistě snížit důsledným zaváděním „na důkazech založených“ (evidence-based) preventivních postupů a včasnou terapií přednádorových stavů. Počet nádorových onemocnění by mohl být snížen o více než 30% důslednou eliminací rizikových faktorů, mezi něž patří: kouření, nadváha či obezita, snížený příjem ovoce a zeleniny, nízká fyzická aktivita, užívání alkoholu, sexuálně přenášené HPV infekce, znečištěni ovzduší polétavým popílkem či výfukovými plyny. Hlavní preventivní principy spočívají v eliminaci výše zmíněných rizikových faktorů, očkování proti HPV a HBV, snižování pracovních rizik a ve snížení expozice slunečnímu záření [9].

V druhé řadě je velmi důležitá časná detekce nádorových procesů. Odhaduje se, že až 1/3 všech malignit by mohla být vyléčena, pokud by byla odhalena a léčena v časných stadiích, zejména před vytvořením vzdálených metastáz. Včasná detekce je založena zejména na: 1) edukaci laické společnosti rozeznat časné příznaky a vyhledat lékařskou pomoc a 2) screeningových programech zachycujících časné příznaky onemocnění.

V konfrontaci s uvedenými daty je zajímavý další údaj WHO [10], kdy eliminace pěti hlavních rizikových faktorů z hlediska mortality obyvatelstva (dětská podvýživa, nechráněný sex, konzumace alkoholu, používání nevyhovující vody a vysoký krevní tlak), zodpovědných za přibližně 25% všech celosvětových umrtí, by prodloužila střední délku života asi o 5 let. Oproti tomu nalezení účinné terapie na léčbu všech onkologických chorob by vedlo k prodloužení střední délky života o pouhé 2 roky.

Spinocelulární karcinomy hlavy a krku

Nádory hlavy a krku jsou jednou z nejčastějších malignit, spinocelulární karcinomy (**HNSCC** - Head and Neck Squamous Cell Carcinoma) jsou šestým nejčastějším onkologickým onemocněním [11]. Celosvětově je udávána incidence cca 563 tisíc případů za rok (data z roku 2002), z nichž 274 tisíc jsou nádory dutiny ústní, 159 tisíc nádory hrtanu, 52 tisíc nádory orofaryngu a 301 tisíc pacientů v souvislosti s tímto onemocněním zemře [12]. Léčebné výsledky se v posledních 20 letech příliš nezlepšily, přes veškeré pokroky v chirurgické i onkologické léčbě. Cesta ke zlepšení terapeutických výsledků povede pravděpodobně přes lepší porozumění biologii a molekulární patofyziologii nádorových onemocnění a jejich interakcím s imunitním systémem. V posledních několika letech došlo vlivem jak významných pokroků v základním výzkumu, tak i v experimentální onkologii k nalezení řady možností potenciálního ovlivnění nádorových buněk a část z nich snad přinese i očekávaný zlom do klinické praxe.

Jednoznačně největší průlom z oblasti experimentální do reálného klinického využití zaznamenala biologická léčba. Jednou z nejvíce studovaných signalizačních drah je tyrosin-kinázová aktivita receptoru pro epidermální růstový faktor (EGFR – Epidermal Growth Factor Receptor). Monoklonální protilátka namířená proti extracelulární části tohoto receptoru – Cetuximab – je v dnešní době běžně užívána v klinické praxi a indikace jejího využití se stále rozšiřují. Zároveň se stále objevují další monoklonální protilátky namířené proti EGFR. Bohužel, ačkoliv drtivá většina HNSCC exprimuje velké množství tohoto receptoru, dochází u části pacientů k selhání této cílené terapie. Zatím neexistuje molekulární marker, který by predikoval úspěšnost terapie. Možné, v budoucnu využitelné markery predikující odpověď na terapii směrovanou na EGFR jsou například amplifikace EGFR genu, KRAS/HRAs mutace, mutace v aktivační cestě PI3K-AKT, EGFR polymorfizmus a dále i polymorfizmus v receptoru FCγRIIa a FCγRIIIa, který je zodpovědný za rozpoznávání a vazbu Fc fragmentu cetuximabu [13]. Povzbudivé jsou výsledky klinických studií používajících

kombinovanou biologickou léčbu směrovanou jak na EGFR tak i na kinázy Src rodiny [14].

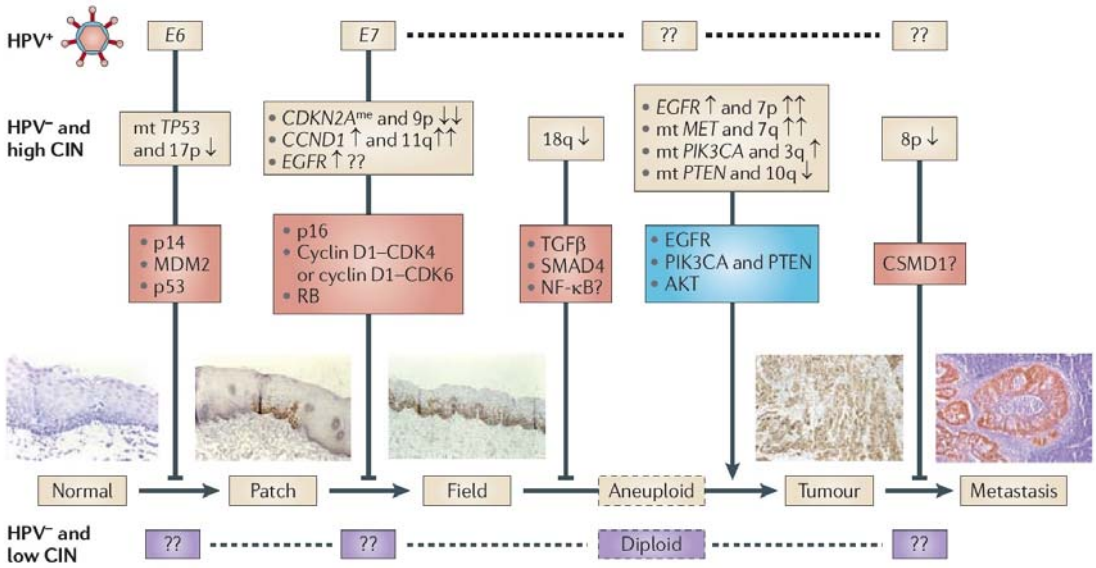
Obdobně jako nastal posun v terapii HNSCC dochází ke změně přístupu i v oblasti diagnostiky a stagingu. Vzhledem k neuspokojivé výpovědní hodnotě klasických klinicko-patologických faktorů je přesnější molekulární klasifikace nezbytná k budoucí přesné a spolehlivé předpovědi terapeutické odpovědi na různé léčebné modality. Fountzilas et al. [15] retrospektivně hodnotili markery jako EGFR, MET, ERCC1 a p-53 (protein i genová exprese) a MMP9 mRNA u pacientů s pokročilým HNSCC léčených radioterapií v konkomitanci s týdenní dávkou cisplatiny a cetuximabu a koreloval jejich hodnoty s odpovědí na léčbu. Pouze hodnota MMP9 byla hodnocena jako prediktivně spolehlivá. Další práce [16, 17] poskytují rozsáhlý přehled celé řady biomarkerů a molekul exprimovaných u HNSCC a jejich vztahu ke klinickému vývoji onemocnění a prognóze pacienta. Retrospektivně bylo hodnoceno množství mRNA pro HER 1-4 (EGFR family) a pro VEGF A, B, C, D a jejich receptorů VEGFR 1, 2, 3 ve vztahu k prognóze. Autoři prokázali vysoký stupeň exprese VEGF-C/VEGFR3 u pacientů s recidivou HNSCC, ať již metastatickým postižením spádových uzlin, tak i šířením do měkkých tkání krku, což taktéž zásadně ovlivňovalo přežití pacientů [17].

Snahou celé řady vědeckých týmů je identifikace dalších molekulárních markerů a potencionálních terapeutických cílů pro nádory hlavy a krku. Jedním z možných je *Akt* (gen pro serine-threonin protein kinázu), jehož exprese a hyperaktivace velmi významně koreluje s progresí HNSCC. Simons et al. [18] prokázali, že je jedna z potenciálních terapeutických možností je kombinace perifosinu, inhibitoru *Akt,* a inhibitorů glutathionového metabolismu. Tím aktivovali v buňkách buněčné linie HNSCC apoptózu prostřednictvím indukce oxidativního stresu. Druhou oblastí v popředí zájmu experimentální onkologie jsou nukleární receptory, které se účastní procesu karcinogeneze a jejichž terapeutické užití je na dosah v podobě probíhajících preklinických a klinických studií [19].

Řada prací v poslední době předkládá důkazy o existenci kmenových nádorových buněk, subpopulace zodpovědné za vznik a růst nádorů, HNSCC nevyjímaje. Tyto nádorové kmenové buňky jsou zodpovědné za rezistenci vůči léčbě a za agresivní a metastatické chování nádorů. Obdobně jako u jiných typů nádorů, včetně solidních, je možné získat subpopulaci nádorových buněk vykazujících vlastnosti podobné jako hematopoetické kmenové buňky pomocí průtokové cytometrie – sortování [20]. Tato metoda otevírá možnosti studia rozdílného biologického chování a následně i obrovské terapeutické možnosti v případě přímého ovlivnění této subpopulace. Nádorové kmenové buňky jsou ovlivňovány ve svém okolí – nádorovém mikroprostředí – celou řadou faktorů. Poměrně hodně je již známo o vlivu nádorových fibroblastů [21-24] umožňujících například epitelo-mezenchymální transformaci (EMT) nádorových buněk [25, 26].
V mikroprostředí se také uplatňuje vliv imunokompetentních buněk vcestovaných do nádoru, až již s cílem podílet se aktivně na efektivní protinádorové odpovědi nebo na základě chemotaktického působení nádoru ve snaze využít funkčních vlastností buněk imunitního systému ve svůj prospěch (Treg, TAM) [27-31].

V literatuře je popisovaná celá řada abnormalit v imunitním systému pacientů s HNSCC. Podrobněji se dále v textu budu věnovat významu regulačních T lymfocytů (Treg), zde se jen zmíním, že jsou v periferní krvi pacientů s HNSCC signifikantně zvýšené oproti zdravým kontrolám, což je v souladu s pracemi popisujícími situaci u jiných typů solidních nádorů [32-36]. Dále byly popsány změny rovnováhy, absolutního počtu i procentuálního zastoupení $CD8^+$ i $CD4^+$ lymfocytů, přetrvávající měsíce až roky po úspěšném ukončení onkologické terapie [37, 38]. Počty NK buněk, jednoho z nejdůležitějších hráčů na poli přirozené protinádorové odpovědi, jsou v periferní krvi snížené, zároveň jejich funkční testy vykazují značné odchylky. Tento fakt koreluje s horší prognózou pacientů s HNSCC [39, 40]. Zvýšené hladiny B lymfocytů ($CD19^+$) byly popsány v mikroprostředí epiteliálních karcinomů [41], v onkologii hlavy a krku bylo prokázáno statisticky významné snížení B lymfocytů v periferní krvi pacientů s metastázami do krčních lymfatických uzlin [39].

Bohužel zatím pouze hudbou budoucnosti je využití imunoterapie nádorů, přestože i v oblasti hlavy a krku přinesly experimenty řadu pozoruhodných výsledků [42, 43]. Základní principy, užívané postupy a výsledky jsou přehledně shrnuty v práci Rapidise [44]. Se zaměřením na tuto část probíhají klinické studie využívající podávání IFN-alfa, pegylovaného IFN-alfa 2, IL-2, IL-12 i rekombinantního fuzního proteinu s částí IL-2. Dále je možné do oblasti imunoterapie v širším slova smyslu zařadit i poměrně širokou škálu používaných monoklonálních protilátek (cetuximab, bevacizumab, anti-CD45 nebo anti-CAE Mab). A v neposlední řadě je nutné zmínit i vakcinační studie, zaměřené na antigeny asociované s HPV, s EBV nebo na CEA [44].



Obrázek 1 - Schéma integrovaného modelu molekulární karcinogeneze pro spinocelulární karcinomy hlavy a krku. Převzato z Leemans at al., 2011 [45].

Tento model znázorňuje tři klíčové kroky, ke kterým musí v průběhu karcinogeneze dojít: 1) přechod od jedné mutované kmenové buňky ke skupině či klonu nádorových kmenových buněk, které začnou nekontrolovatelně růst, 2) plošný růst přechází v invazivní karcinom, a 3) metastatický rozsev. Konkrétní molekulární mechanizmy se liší u HPV pozitivních a negativních nádorů.

Karcinomy orofaryngu

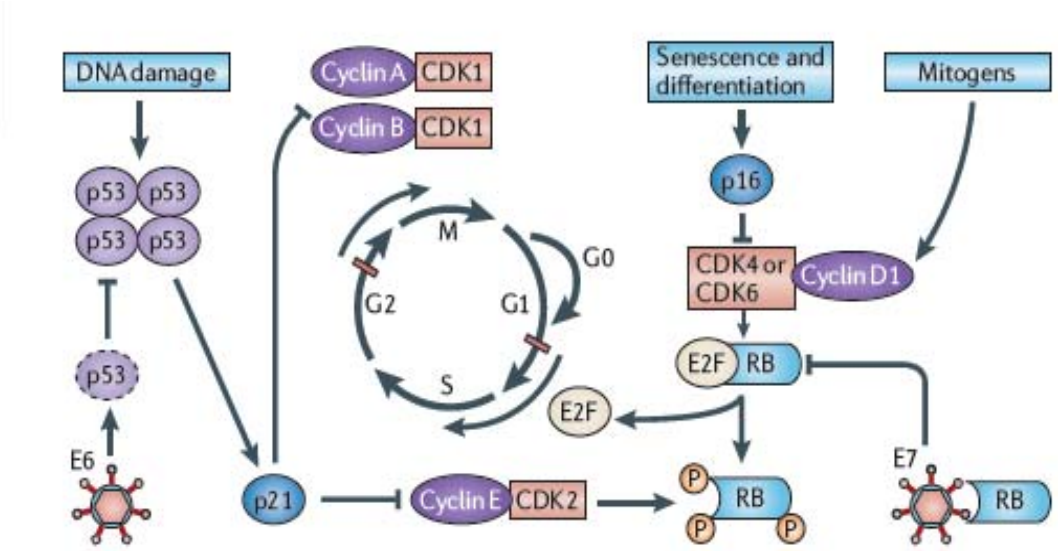
Jednou z nejčastějších lokalit postiženou dlaždicobuněčným karcinomem v oblasti hlavy a krku je orofarynx. Řada těchto případů je způsobena nejčastějším rizikovým chováním – kouřením a intenzivní konzumací alkoholu, které vedou v průběhu času k řadě mutací klíčových genů regulujících zejména buněčný cyklus. Jak prokázala řada studií v posledních letech [46-50], dalším významným nezávislým rizikovým faktorem ovlivňujícím vznik a rozvoj nádorů v oblasti hlavy a krku je infekce lidským papillomavirem (HPV). Význam HPV pro ano-genitální nádory je znám již poměrně dlouho, v onkologii hlavy a krku se objevuje až v posledních asi 10 letech [48]. Je známo více než 100 typů HPV, ale pouze asi 15 z nich vykazuje onkogenní potenciál [51]. U nádorů hlavy a krku je nejčastěji detekován HPV 16 (více než 90%). U nádorů orofaryngu (zejména u nádorů tonzil a kořene jazyka) je přítomnosti HPV prokazována až ve 40 – 80% případů (údaje se však pohybují od 10 do 90%) [49, 52]. V Evropě je procento orofaryngeálních nádorů způsobených HPV nejvyšší ve Švédsku (až 90%), naopak v zemích s vysokým zastoupením kuřáků v populaci, například v Itálii, je nižší než 20% [52].

Velké epidemiologické studie prokazují v mezinárodním měřítku nárůst incidence orofaryngeálních nádorů. Například v USA stoupla incidence nádorů tonzily v rozmezí let 1974 až 2002 u žen 3,5x a u mužů 2,6x. Zvyšující se incidence HPV-asociovaných orofaryngeálních karcinomů je považováno za alarmující virový nádorový epidemiologický faktor [53]. Tím, jak se snižuje procento kuřáků, proporcionálně narůstá význam HPV a zároveň se zvyšuje podíl karcinomů orofaryngu ze všech nádorů hlavy a krku. Například v USA se zvýšil z 18% v roce 1973 až na 31% v roce 2004. Práce ze Švédska prokázaly nárůst HPV-pozitivních HNSCC z 23% v roce 1970 na 57% v roce 1990 a až na 93% v roce 2007 [54]. HPV je dnes považován za primární příčinu spinocelulárních karcinomů tonzily v Evropě a v Severní Americe [52].

Je prokázáno, že HPV pozitivní nádory jsou diagnostikovány u mladších pacientů, většinou bez klasických rizikových faktorů jako je kouření a konzumace alkoholu [46, 55]. Práce z USA prokazují tento typ onemocnění zejména u bělochů mužského pohlaví. Jelikož je HPV sexuálně přenosná infekce není překvapující, že posun k mladším věkovým skupinám je vysvětlitelný změnami sexuálních norem, zejména nárůstem orálních sexuálních partnerů a posunem do mladších věkových skupin. Obdobně je změnami v sexuálním chování vysvětlován i nárůst zejména u mužů ve srovnání se staršími generacemi. Vyšší prevalence HPV v tkáních ženského pohlavního ústrojí může při nárůstu orálních sexuálních partnerů vysvětlovat nárůst HPV-pozitivních nádorů u mužů [52]. Incidence, a tedy i význam HPV pozitivních HNSCC se postupně zvyšuje v posledních 10 letech [55]. Tento parametr významně ovlivnil chápání etiopatogeneze nádorů orofaryngu a význam HPV je proto stále předmětem intenzivního zájmu lékařů a vědců. HPV-pozitivita HNSCC je důležitým prognostickým faktorem, protože deklaruje vyšší senzitivitu nádorových buněk k radio- i chemoterapii [52]. HPV-asociované nádory se svými charakteristikami výrazně liší od HPV-negativních HNSCC a v dnešní době se intenzivně diskutuje, zda je stále možné mluvit o tomtéž onemocnění [56]. Biologická odlišnost HPV pozitivních HNSCC spočívá zejména v degradaci p53, inaktivaci dráhy pRb a v upregulaci p16. HPV-pozitivní nádory orofaryngu většinou vykazují dysregulaci kontroly buněčného cyklu, zejména prostřednictvím pRb a cyklinu D1, které jsou naopak zvýšeně exprimovány u HPV-negativních orofaryngeálních karcinomů [46, 56]. E7 onkoprotein vytváří komplexy s nízce fosforylovanou formou onkosupresorového proteinu pRb , což vede ke snížení hladiny pRb na buněčné úrovni, a to vede k uvolňování E2F transkripčního faktoru zasahujícího do buněčného cyklu. Tím je zpětně indukovaná overexprese CDKN2A/p16, což je inhibitor komplexu D/cyklin-dependentní kinázy, která fosforyluje pRb v HPV negativních nádorech. Tímto je zablokována progrese buněčného cyklu [57]. Dysfunkce jednoho z nejznámějších onkosupresorových proteinů p53 je prokázána u většiny HNSCC, molekulární podstata této poruchy se však liší u HPV pozitivních a HPV negativních nádorů. Zatímco u HPV negativních je ve více než 50% podmíněna mutací genu, u HPV-pozitivních je naopak p53 vázán virovým onkoproteinem E6 a tím je spuštěna jeho degradace [46].

Nádory vykazující vysokou aktivitu HPV mají lepší prognózu než nádory s nízkým virovým loadem či které jsou HPV negativní [46, 56]. Pacienti s HPV-pozitivními nádory, přestože mají častěji lokoregionální metastatické postižení lymfatických uzlin [56], lépe odpovídají na chemoterapii, radioterapii i chirurgickou léčbu a vykazují aktivaci imunitního systému proti HPV antigenům [58]. Biologická podstata těchto rozdílů však zatím není jasná.

Zdá se, že HPV mají nějakou zvláštní afinitu vůči tkáni tonzil a kořene jazyka, podmíněnou pravděpodobně typy exprimovaných receptorů. Tonzilární krypty jsou morfologicky obdobné transformační zóně cervixu uteri, buňky uložené v basální vrstvě jsou citlivé na možnost ovlivnění pomocí HPV [59, 60]. Proto také klasické histologické vyšetření HPV-pozitivního nádoru je často popisováno jako nádor s basaloidními rysy. Metastázy do lymfatických uzlin bývají cystické [61, 62]. Klinicky jsou HPV-pozitivní nádory nejčastěji diagnostikované jako časná T stádia, ale s pokročilým N stádiem, obvykle v III a IV klinickém stádiu.



Obrázek 2 - Deregulace buněčného cyklu HPV infekcí. Převzato z Leemans at al., 2011 [45].

Buněčný cyklus je regulován komplexy cyklinu a cyklin-dependentních kináz (CDK) a zároveň přítomností a funkcí jejich inhibitorů. Přechod přes G1 kontrolní bod buněčného cyklu je kontrolován pomocí RB proteinů (retinoblastoma pocket proteins), p107 (=RBL1) a p130 (=RBL2). Ty se za normálních okolností váží na inaktivní E2F transkripční faktor, který indukuje expresi genů S fáze. Pokud přijde transkripční signál, pak je aktivován komplex cyklinu D1 a CDK4 nebo CDK6. Komplex fosforyluje RB proteiny a tím uvolní, tedy aktivuje E2F transkripční faktor. Ten indukuje cyklin E a zároveň fosforylace RB pomocí komplexu cyklinu E a CDK2 spustí přechod do

S fáze. Inhibitorem komplexu cyklinu D1 a CDK4 a CDK6 je p16INK4A, který je kódován CDKN2A, genem lokalizovaným v INK4A lokusu na chromozómu 9p21. Exprese p16INK4A ovlivňuje stárnutí a diferenciaci buněk. Rovnováha mezi cykliny, CDK a jejich inhibitory určuje, zda je překročen kontrolní bod buněčného cyklu, většinou je k tomuto kroku nutný signál některým z růstových faktorů. Druhý důležitý kontrolní bod buněčného cyklu je v G2 fázi, kdy proběhne replikace DNA a jsou opravovány zjištěné chyby. Klíčový protein, exprimovaný v návaznosti na zjištěné chyby je p53, za normálních okolností je ale udržován v nízké koncentraci degradací pomocí MDM-2. Je-li zjištěno poškození DNA, pak je zvýšena fosforylace kontrolních kináz CHK1 a CHK2, což vede ke zvýšení aktivity p53 díky degradaci řady dalších inhibičních molekul. Tetramerní forma p53, působící jako stresový transkripční faktor, indukuje expresi $p21_{cip}$ (CDKN1A), který následně inhibuje řadu komplexů cyklin-CDK a tím zastaví buněčný cyklus. Kromě této klíčové role v regulaci buněčného cyklu je p53 taktéž jedním z hlavních regulátorů apoptózy a dalších stresem vyvolaných buněčných funkcí. Genom HPV obsahuje celou řadu časně i pozdně exprimovaných genů a kóduje dva virové onkoproteiny, E6 a E7. E6 protein váže p53 a tím způsobí jeho degradaci, E7 protein váže a inaktivuje RB. Dopady exprese těchto dvou onkoproteinů na molekulární úrovni jsou aktivace buněčného cyklu a inhibice apoptózy způsobené díky p53, což ve výsledku vede k replikaci HPV. V produktivní fázi infekce je exprese E6 a E7 potvrzována v diferencujících se vrstvách dlaždicového epitelu a jsou produkovány viriony HPV. Onkogenní fáze infekce je spojená s expresí E6 a E7 v bazální vrstvě (jsou zde přítomny i kmenové buňky) a působí zde rozložení kontrolních bodů buněčného cyklu.

Diagnostika HPV

Pro odhadnutí vývoje onemocnění, při zahrnutí všech ostatních prognózu ovlivňujících aspektů (časná detekce či pokročilý nádor, lokalizace primárního nádoru, typ zvolené léčby), se dnes HPV detekce ukazuje jako spolehlivý biomarker [63-67]. To zároveň zvyšuje potřebu snadné, spolehlivé, vysoce přesné a jasně interpretovatelné metody k diagnostice HPV v klinické praxi. Přestože i klinické doporučené postupy [68] zmiňují rutinní HPV detekci u nádorů orofaryngu, není zatím jasně definovaná nejlepší diagnostická metoda.

V současnosti je užívána řada technik, od klasické PCR, přes RT-PCR kvantifikující virovou nálož, přes typově specifickou DNA in-situ hybridizaci, přes detekci sérových protilátek proti řadě epitopů až k imunohistochemické detekci přidružených biomarkerů jako například p16. Ačkoliv PCR detekce E6 HPV onkogenu ze zmrazených řezů je dnes obecně doporučována jako zlatý standard pro stanovení přítomnosti HPV, výběr metody vhodné pro rutinní klinické použití bude ovlivněn zejména senzitivitou, specificitou, snadnou interpretovatelností výsledků, cenou a snadností provedení. Vývoj nefluorescenčních chromogenů

umožnil vizualizaci DNA hybridizace pomocí klasického světelného mikroskopu, navíc vypracování techniky pro značení formalinem a parafinem fixovaných řezů udělalo tuto metodu srovnatelnou a použitelnou v průběhu klasického zpracování histologických vzorků a umožnilo retrospektivní zpracování archivovaných tkáňových bločků. In-situ hybridizace umožňuje přímou vizualizaci distribuce HPV v tkáni nádoru. Lokalizace HPV genomu v jádrech nádorových buněk nám umožní zhodnotit etiologicky relevantní HPV infekci (klonální přítomnost ve všech nádorových buňkách) a rozlišit náhodnou přítomnost či kontaminaci (přítomnost malého množství kopií pouze v malém množství buněk). Zvýšení sensitivity in-situ hybridizace nezhoršuje specificitu. Zavedení různých kroků vedoucích k amplifikaci signálu umožnilo zvýšení sensitivity této techniky až na úroveň záchytu jedné virové kopie v buňce.

Naproti tomu detekce kvantitativní PCR umožňuje rozlišit mezi transkripčně aktivní (klinicky relevantní) a transkripčně inaktivní infekcí, což je pro klinické využití jistě nutný krok.

Jak bylo již zmíněno, v HPV pozitivních nádorech orofaryngu inaktivuje virový onkoprotein E7 funkci produktu *RB* genu, což způsobuje značné poruchy v klíčových bodech této metabolické cesty. Tím je indukována zvýšená exprese p16 až na imunohistochemicky detekovatelné hodnoty [28, 69]. Proto je také často p16 označován jako alternativní marker pro HPV infekci v orofaryngeálních karcinomech [70, 71]. V řadě studií byla na velkém počtu pacientů s orofaryngeálním HNSCC prokázána vysoká shoda v imunohistochemické detekci p16 a HPV-16 pomocí in-situ hybridizace (93%). Diskrepance v podobě imunohistochemicky detekovaného p16 a zároveň negativního HPV-16 v in-situ hybridizaci [52] mohou být dány malým podílem dalších typů HPV (ne pouze 16, udáváno v 5-10%) na nádorech orofaryngu a dále i mutací v pRB metabolismu z jiných než HPV příčin (například mutační inaktivita retinoblastomového proteinu). Jako nezvratný důkaz podílu HPV infekce na vzniku HNSCC v orofarygu je považována detekce mRNA pro E6 a E7. Je-li imunohistochemická detekce p16, jakožto alternativní metoda, porovnána s touto metodou, vykazuje pak 100% senzitivitu, ale jen 79% specificitu [72]. Některé práce naznačují, že p16 overexprese by se mohla stát prediktorem klinického průběhu nezávislým na

detekci samotného HPV. Většinový názor se v současné době kloní spíše ke kombinaci dvou nezávislých metod, z nichž jedna by měla být IHC detekce p16.

Velice dobře proveditelná je kombinace imunohistochemické detekce p16 a HPV in-situ hybridizace. Vzhledem k téměř 100 % senzitivitě je vhodná jako první metoda IHC p16, která eliminuje HPV negativní případy. Naopak s vysokou specificitou (téměř 100%) je použitím HPV in-situ hybridizace sníženo procento falešně pozitivních případů. Případy p16 pozitivní a HPV 16 negativní jsou řazeny do zvláštní skupiny non-HPV 16, kde se předpokládá podíl jiného typu onkogenního viru. Pro detailní analýzu této skupiny je následně možné použít některou ze specializovaných metod, jako je širokospektrá in-situ hybridizace nebo některé z PCR metod [72]. Nicméně nezávisle na dalších zmiňovaných metodách, použití IHC p16 a HPV 16 in-situ hybridizace přesně stanoví HPV status většiny karcinomů orofaryngu.

Klinicky relevantní HPV infekci je také možné detekovat pomocí kombinace IHC p16 a detekce protilátek proti E6/E7 antigenu, což je kombinace vysoce specifická i senzitivní, v běžné praxi dobře dostupná [73].

HPV in-situ hybridizace a IHC p16 jakožto praktický diagnostický přístup k ověření HPV statusu by mohly být použity i pro vyhodnocení cytologických preparátů, včetně FNAB [63, 74]. Další rozšiřování HPV detekce v krvi i v dalších tělesných tekutinách jistě posílí postavení HPV jakožto klinicky významného biomarkeru. PCR detekce HPV DNA v krvi [75] či ve slinách [76] u pacientů po léčbě HPV-pozitivního nádoru by mohly sloužit jako marker recidivy onemocnění. Zároveň také hladiny protilátek proti řadě s HPV asociovaných epitopů mohou predikovat HPV status a jsou někdy snadno dostupným a detekovatelným markerem k monitoraci léčby a vývoje onemocnění bez nutnosti získání vzorků tkáně [75, 77].

HPV status je významný prognostický faktor pro celkové přežití, pro „progression-free survival“ a může být i prediktivní marker pro odpověď na léčbu u nádorů orofaryngu a pravděpodobně i u dalších lokalit v oblasti hlavy a krku. In-situ hybridizace je efektivní metoda proveditelná ve většině patologických laboratoří a

IHC p16 je použitelná alternativní metoda. Postupovat v obráceném pořadí doporučují někteří autoři [78], kteří jako selektivní metodu preferují IHC p16 a následně HPV DNA specifikaci pomocí FISH či PCR. Argumentem pro tuto kombinaci je vysoká senzitivita i specificita.

HPV-pozitivní nádory mají nižší pravděpodobnost loko-regionální recidivy. Pravděpodobnost vzdáleného metastazování není přítomností HPV infekce ovlivněna. Kouření zvyšuje pravděpodobnost recidivy a snižuje procento celkového přežití u HPV pozitivních nádorů, a některé studie již dle kombinace těchto faktorů stratifikují pacienty do skupin podle velikosti rizika [79].
HPV-pozitivní skupina nádorů orofaryngu je zvláštní klinická i biologická skupina s řadou doposud nezodpovězených otázek. Další studie budou muset osvětlit, proč se například incidence tohoto onemocnění zvyšuje ve skupině mladých mužů, bělochů. Vypracování lepší HPV diagnostiky s detekcí počtu HPV DNA kopií (viral load) umožní pochopení významu tohoto faktu pro biologii HPV pozitivních nádorů a pro celkové přežití či časnou recidivu onemocnění, neboť již dnes je prokázáno, že terapeutické odpověď je úměrná počtu virových kopií [80].
Zároveň se otevírá otázka primární a sekundární prevence, významu HPV vakcinace, včetně použití terapeutických HPV vakcín jakožto adjuvantní formy léčby u lokálně pokročilých či metastazujících stádií onemocnění.
Na základě uvedených výsledků (definování HPV pozitivních nádorů jako nové entity) bude nutné změnit design klinických studií zařazením HPV diagnostiky a na tomto podkladě přesně stratifikovat riziko a definovat léčebné strategie zatížené menším rizikem. Zároveň bude asi nutné prodloužit dispenzarizaci pacientů k lepšímu porozumění biologické podstaty samotného onemocnění a zejména biologickým důvodům v případě selhání léčby [52].

Protinádorová imunita

V této kapitole nebude zmíněn úplný výčet efektorových mechanizmů využívaných imunitním systémem k rozeznání a eliminaci nádorových buněk, ale jsou vybrány některé zajímavé aspekty, související zejména s regulací či modifikací charakteru imunitních reakcí.

Problematika protinádorové imunity stojí vlastně na dvou principiálních otázkách:
1) jak může imunitní systém nádor rozpoznat a

2) jaké prostředky využívají nádorové buňky k obraně před imunitním systémem jedince (ať již aktivně v podobě ovlivňování svého okolí sekrecí cytokinů či jiných působků (solubilní FasL) nebo pasivně v podobě snížené či zvýšené exprese povrchových molekul (např.snížené MHC-I nebo naopak zvýšené exprese FasL).

Identifikace

Imunitní systém může rozeznat nádorové buňky dvěma způsoby, buďto pomocí pro nádor specifických antigenů (TSA, molekul, které jsou exprimované pouze nádorovými buňkami) anebo pomocí takzvaných s nádorem asociovaných antigenů (TAA, molekul, které jsou exprimovány v rozdílné míře na buňkách nádorových a na buňkách normálních) [81]. Antigeny pro nádor specifické jsou produktem genů původně normálních buněk změněné nějakou unikátní mutací [82]. Antigeny detekovatelné na povrchu buněk mohou (ale nemusí) souviset s patogenezí daného onemocnění. Například pokud je nádorové onemocnění podmíněno virovou infekcí, mohou pak být virové antigeny detekovány i na povrchu nádorových buněk. Příkladem jsou produkty E6 a E7 genů lidského papillomaviru (HPV), který se kauzálně podílí na vzniku karcinomu čípku i karcinomů v oblasti orofaryngu, nebo jaderné antigeny viru Epstein-Barrové (EBNA-1) exprimované Burkittovým či Hodgkinovým lymfomem a karcinomem nosohltanu [83, 84].

Přestože u řady nádorových onemocnění víme o rizikových faktorech, přesná etiopatogeneze je stále ještě nezodpovězenou otázkou. U některých malignit se

v pochopení jejich vzniku pokročilo teprve až po izolaci některých exprimovaných antigenů. Tento pokrok byl umožněn zejména rozvojem hybridomových technologií a produkcí monoklonálních protilátek [85]. V myších systémech produkované monoklonální protilátky rozpoznávající lidské antigeny jsou schopné přesně nalézt svůj epitop a tím odhalit charakter detekované buňky [86].

Rozvoj metod umožňující *in vitro* propagaci T lymfocytů, zejména tedy nádorově specifických T lymfocytů, vedl k velmi důležitému průlomu, identifikaci MAGE-1, antigenu specifického pro melanom, který je rozpoznáván lidskými T buňkami [87]. Tento poznatek spustil hledání dalších a dalších nádorově specifických antigenů, které mohou být potenciálním terapeutickým cílem [88]. Prezentace peptidových antigenů na pozadí MHC I molekul na povrchu antigen prezentujících buněk (APC), které jsou rozpoznávány CD8+ lymfocyty je všeobecně známým mechanizmem. Obdobně jsou prezentovány i nádorové antigeny, ať již proteiny, produkty mutovaných genů, nebo normální proteiny exprimované na nádorových buňkách v abnormální míře [89-92]. (viz. Obrázek 3)

Dalším technologickým pokrokem byla jistě možnost propagovat *in vitro* dendritické buňky a následně je pak využít k obnovení a zesílení imunitní odpovědi [93]. Prostřednictvím kokultivace s dendritickými buňkami byla identifikována celá řada nádorových proteinů, například cyklin B1, který je za normálních okolností detekovatelný v jádře při přechodu buňky z G2 do M fáze buněčného cyklu, ale u řady lidských nádorů je naopak zvýšený v cytoplazmě [94]. (viz. Obrázek 4)

Další možností identifikace nádorových antigenů je analýza vzorků krevního séra, kde jsou některé z nich zastoupeny, na rozdíl od vzorků získaných od zdravých dobrovolníků. Takto byl například nalezen antigen NY-ESO-1 [95].

Nádorových antigenů byla do současnosti popsána celá řada, při úvahách o jejich budoucím terapeutickém využití bylo klíčové určit bezpečná kritéria. Nádorový antigen vybraný jako terapeutický cíl musí být natolik specifický, že po jeho nalezení je eliminována pouze cílová nádorová buňka, nikoliv okolní zdravá tkáň. V preklinickém testování jsou např.vakcíny proti TAA CEA, MUC1 a Her2/*neu*

[96-98]. Za druhé je důležitá stabilita exprese daného antigenu. Antigen musí být exprimován na většině nádorových buněk bez ohledu na jejich aktuální stav.

Imunogenicita

V minulosti bylo předpokládáno, že nádorové antigeny nemohou u lidí aktivovat účinnou imunitní reakci, protože neindukují protektivní zánět. Tento předpoklad však byl vyvrácen celou řadou studií.

Pod vlivem léčby a vlivem dalších faktorů v nádorovém mikroprostředí je možné rozdělit apoptotickou smrt nádorových buněk na tzv. imunogenní a neimunogenní. Neimunogenní smrt buňky spočívá v indukci apoptózy bez následující imunitní reakce. Naopak imunogenní smrt nádorové buňky (vyvolaná například antracyklinovými cytostatiky, paklitaxelem, cyklofosfamidem, deriváty platiny či roentgenovým či ultrafialovým zářením) je vyvolaná intracelulárními molekulárními procesy jako je translokace kalretikulinu a Heat Shock Proteinů – tzv.„eat me“ signalizace - doprovázenými uvolněním alarminů typu HMGB1 – tzv.“danger signal“. Toto vede k maturaci dendritických buněk, které pak jako APC iniciují imunitní reakci [96, 97, 99-104]. Některá moderní protinádorová léčiva navozují imunitní odpověď doprovázenou vznikem dlouhodobé imunologické specifické protinádorové paměti (podmíněné nejčastěji CD8+ CTL) [105].

Produkty onkogenů, které jsou aktivovány v časných fázích onkogeneze jsou schopny velmi silně ovlivňovat imunitní reakci. Například plicní nádory, které jsou iniciovány mutací K-ras onkogenu, produkují chemokiny atrahující do nádorového mikroprostředí imunokompetentní buňky [106]. Dalším příkladem může být RET-PTC protein, produkt fúze dvou onkogenů, který se významně podílí na rozvoji papilárního karcinomu štítné žlázy. RET-PTC mění aktivitu transkripčního jaderného faktoru κB (NF-κB), který kontroluje produkci imunoregulačních cytokinů. RET-PTC zvyšuje produkci GM-CSF (granulocyte–macrophage colony-stimulating factor) a monocytového chemotaktického proteinu 1, čímž spoluvytváří prozánětlivé prostředí [107]. Dále i nádorové antigeny jako MUC1, CEA a NY-ESO-1 jsou schopné atrahovat působky vrozené imunity a mohou být rozeznány jako tzv. „danger signals“ [96, 97, 103, 104].

Imunitní systém a nádor

Základním předpokladem je, že imunitní systém rozeznává antigeny na maligních buňkách jako cizí a snaží se je eliminovat. Hypotézu imunitního dozoru, nebo-li „Cancer immunosurveillance“, vyslovil poprvé Frank MacFarlene Burnet v polovině minulého století (1957, v roce 1960 obdrželi sir Frank MacFarlene Burnet a Peter Brian Medawar Nobelovu cenu za medicínu a fyziologii) [108]. Kontrolní fyziologická funkce imunitního spočívá v rozpoznání a v časné eliminaci všech vzniklých transformovaných nádorových buněk. Tato myšlenka byla kontroverzní až do doby, než bylo naše chápání protinádorové imunity posunuto a upřesněno díky technologickému pokroku a zvířecím modelům. Na myších modelech, které díky genovým manipulacím postrádají některé přesně definované efektorové mechanizmy (např. interferon 1) bylo prokázáno signifikantní snížení schopnosti eliminovat růst nádorových buněk imunitním systémem [98, 106, 109, 110]. Na základě dalších experimentů byl později definován proces setkání tvořícího se nádoru a imunitního systému, dnes nazývaný „immunoediting“ [111]. Zahrnuje tři etapy: 1) eliminaci nádoru, odpovídající původní Burnetově „immunosurveillanci“, 2) stav rovnováhy (equilibrium) a 3) růst nádoru, kdy se nádor vymaní z efektivní protinádorové odpovědi [102, 111].

Z pochopitelných důvodů je experimentální prokázání „imunoeditace“ u lidí velmi komplikované, ale díky řadě prací bylo nepřímo prokázáno, že se na konfrontaci nádoru a imunitního systému podílí jak vrozená, tak adaptivní imunita. Vliv imunosupresivní léčby byl prokázán ve studii, která sledovala 905 pacientů s transplantovanými orgány, plícemi, srdcem nebo obojím. Byla sledována jak pravděpodobnost rejekce orgánu, tak i výskyt nádorových onemocnění. Celkově bylo nově diagnostikováno 102 nádorových onemocnění, což je 7,1 x více než v normální populaci. Hlavním typem byly leukémie a lymfomy (26,2 x více), nádory hlavy a krku (21x více) a nádory plic (9,3x více) [112] . V jiné studii sledovali japonští autoři 11 let 3625 zdravých lidí a prokázali závislost rizika vzniku nádoru po dobu sledování na snížené vrozené cytotoxicitě efektorových

lymfocytů v periferní krvi, především NK buněk (testováno *in vitro* jako ^{51}Cr test proti lidským leukemickým buňkám K562), v okamžiku vstupu do studie [113].

Imunitní systém pochopitelně také ovlivňuje výskyt recidiv nádorového onemocnění. Studie sledující 603 pacientů s kolorektálním karcinomem prokázala pozitivní korelaci prognózy a infiltrace tkáně tumoru T lymfocyty ($CD3^+$), tedy že přítomnost či absence T lymfocytů v resekované nádorové tkání predikuje velmi přesně budoucí klinický vývoj, mnohem lépe než doposud užívané parametry jako velikost nádoru a postižení lymfatických uzlin [114]. Obdobné výsledky byly již prokázány i pro nádory děložního čípku [115], prsu [116], močového měchýře [117] a folikulárního lymfomu [118], včetně spádových lymfatických uzlin [119]. Všechny tyto práce podporují závěr, že hodnocení pacientova imunitního systému celkově, tak i v jednotlivých konkrétních parametrech vztahujících se k danému typu nádorového onemocnění by se mělo stát součástí standardním prognostickým parametrem a mělo by být zahrnuto do terapeutické rozvahy.

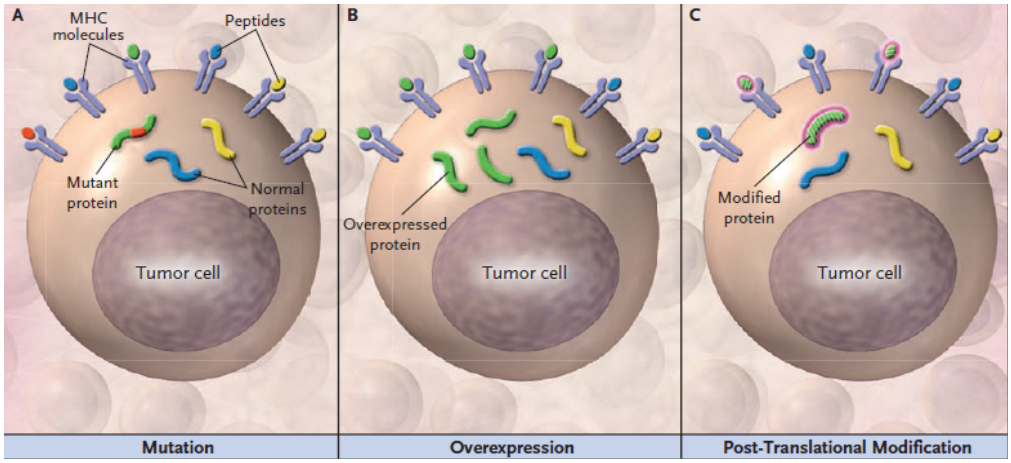
Regulace imunitního systému ve vztahu k nádorovým onemocněním

Nádorové onemocnění má schopnost ovlivňovat imunitní systém jak systémově, tak působením v nádorovém mikroprostředí [120] (viz. Obrázek 5). Mezi nejlépe prozkoumané molekuly s imunosupresivním působením patří TGF-β (transforming growth factor-β), interleukin 10 (IL-10) a Fas ligand exprimovaný na povrchu nádorových buněk či uvolňovaný do okolí tumoru [121, 122]. Kromě toho celá řada lidských nádorů produkuje enzym IDO (indolamin-2,3-dioxygenázu), který má výrazné parakrinní imunosupresivní působení [123, 124]. Poprvé byl popsán v souvislosti s tolerancí vůči fetálním antigenům v průběhu těhotenství [125], poté byl identifikován jako jeden z hlavních inhibitorů aktivace T lymfocytů [126].

Hlavní imunoregulační působení v nádorovém mikroprostředí zastávají Treg (regulační T lymfocyty) a TAM (s nádorem asociované makrofágy), které inhibují efektorové T lymfocyty řadou různých mechanizmů, jako je produkce TGF-β či IL-10 (interleukin-10) [30, 127]. Imunosupresivní působení Treg je popsáno i

systémově, prognostický význam byl prokázán u pacientů s nádory hlavy a krku [27, 128, 129] a s melanomem [130]. Více je o této podskupině lymfocytů pojednáno v následující kapitole.

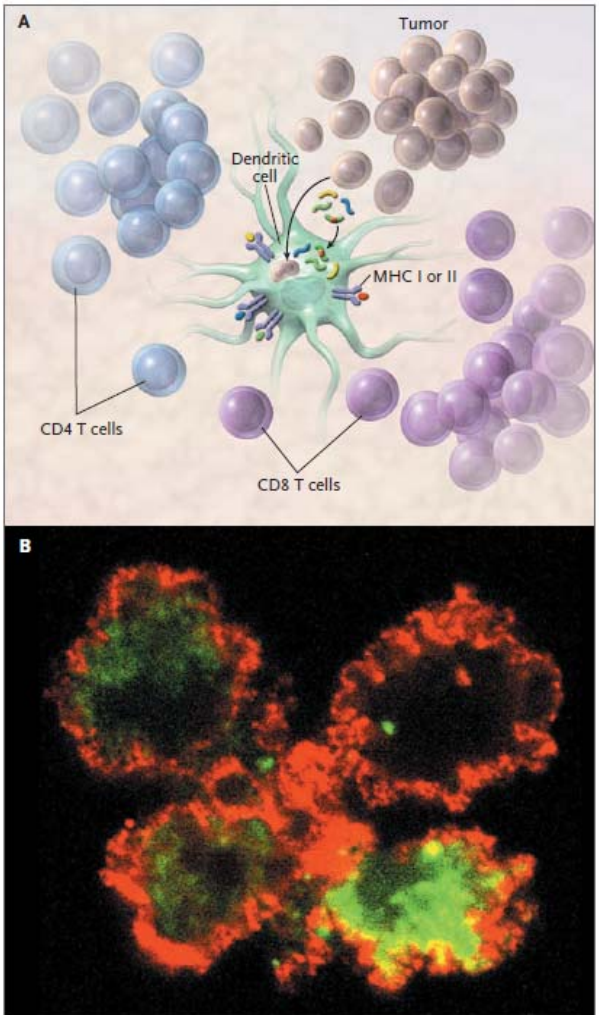
Dalším významným regulátorem nádorového mikroprostředí jsou makrofágy, nazývané TAM (tumor associated macrophages). Makrofágy jsou velmi různorodá a multifunkční součást imunitního systému. Vznikají v kostní dřeni, jako monocyty cirkulují v periferní krvi a dozrávají v tkáních, kam vcestovávají extravazací. Svůj fenotyp mohou měnit podle prostředí, ve kterém se nachází. Jsou popsány dvě základní cesty aktivace makrofágů. M1 aktivace, tzv. klasická, je charakterizovaná produkcí IL-12 a IL-23 a aktivuje Th buňky a APC (buňky prezentující antigeny). V nádorovém mikroprostředí však dochází vlivem kortikoidů, IL4, IL-8, IL-10 a IL-13 k alternativní aktivaci typu M2. M2 inhibují proliferaci i aktivaci T lymfocytů a zároveň velmi účinně stimuluji neoangiogenezi, zejména produkcí proangiogenních faktorů jako je např. VEGF. M2 dále produkují imunosupresivní cytokiny IL-10 a TGFβ, i cytokiny přímo stimulujících růst nádorů, např. EGF. Jejich zvýšené zastoupení koreluje s horší prognózou. [30, 31, 131, 132].



Obrázek 3 - Tři způsoby, jak se mohou vlastní antigeny změnit na nádorové. Převzato z Finn, 2008 [133].

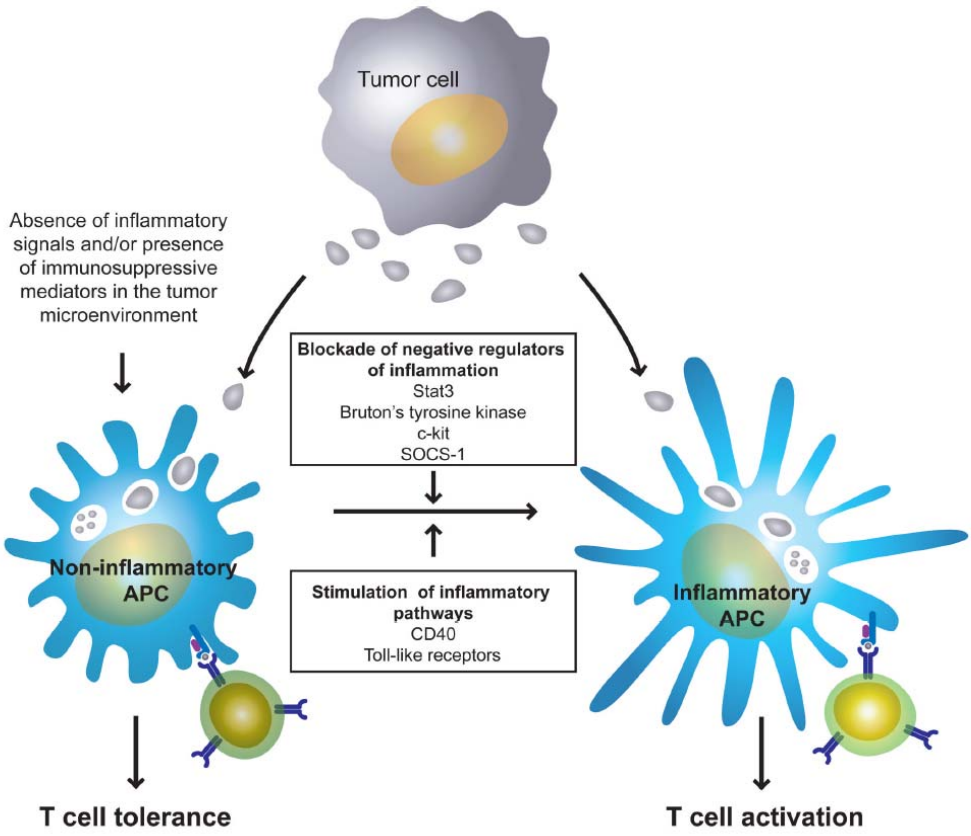
Peptidy ze tří různých proteinů (žlutý, modrý a zelený) jsou prostřednictvím molekul MHC I. či II.třídy prezentované na povrchu buňky. Na obrázku A je znázorněna mutace genu, podmíněna chybnou reparací DNA a následná prezentace produktu tohoto změněného genu na povrchu nádorové buňky. V části B je znázorněna situace, kdy normální protein, peptid, je prezentován na

povrchu nádorové buňky díky chybě či absenci regulačních mechanizmů. V třetí části (C) je znázorněna posttranslační modifikace normálního proteinu (glykosylace, fosforylace, ...), díky níž jsou opět na povrchu nádorové buňky exprimovány abnormální peptidy.



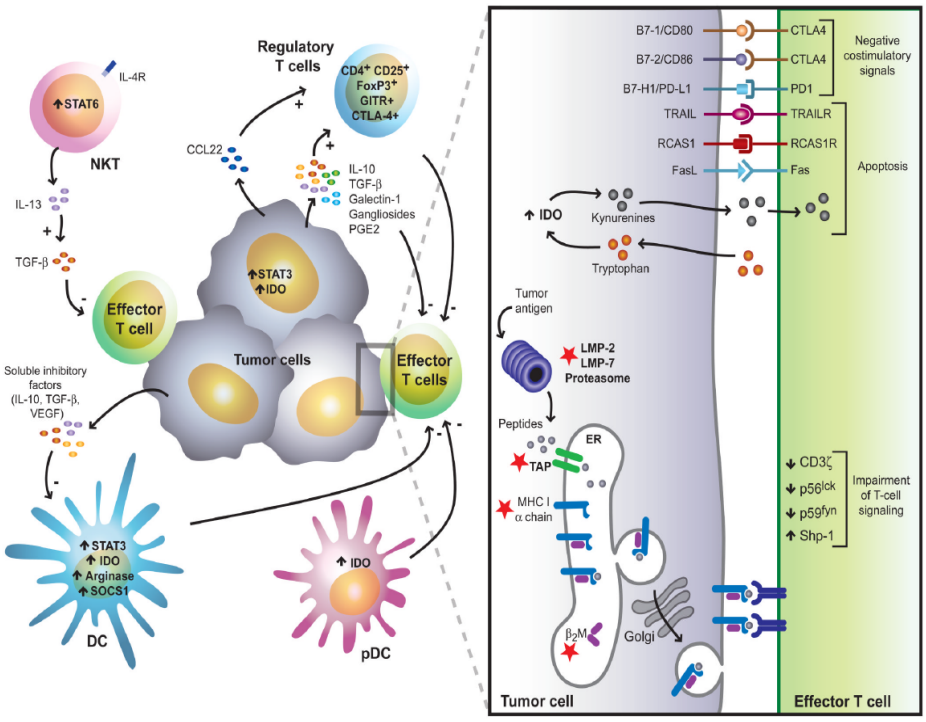
Obrázek 4 – Nádorové antigeny vyvolají T-buněčnou odpověď, pokud jsou prezentovány naivním T lymfocytům na povrchu DC či jiných APC. Převzato z Finn, 2008 [133].

V části A je zobrazená dendritická buňka, která prostřednictvím MHC I a II vystavuje na svém povrchu antigeny získané z rozpadajících se nádorových buněk. Pokud je tato prezentace doplněna vhodnými kostimulačními signály (např.CD28-CD80/CD86; ICOS), stimulují dendritické buňky nádorově specifické CD8+ i CD4+ T lymfocyty. V části B je vidět dendritickou buňku (červeně), která pohlcuje část nádorové buňky (zeleně, exprimuje GFP).



Obrázek 5 - Faktory ovlivňující nádorem indukovanou toleranci a aktivaci. Převzato z Rabinovich, 2007 [120].

Na rozpoznání nádorových antigenů imunitním systémem se podílí APC, které pohltí nádorové buňky, poté migrují do sekundárních lymfatických orgánů, kde prezentují antigeny nádorově specifickým T buňkám. Pokud chybí prozánětlivá stimulace, kostimulace či jsou v nádoru přítomny imunosupresivní faktory, může tento proces vést ke vzniku tolerance.



Obrázek 6 - Mechanizmy využívané nádory k vymknutí se imunologické kontrole. Převzato z Rabinovich, 2007 [120].

Nádorové buňky využívají řadu imunosupresivních mechanizmů, které tlumí efektivní imunitní reakci. Patří mezi ně narušování TCR signalizace, blokace prezentace nádorových antigenů pomocí APC, aktivace negativních kostimulačních signálů v rámci nádorového mikroprostředí (CTLA-4/B7, PD-1/PD-L1), vytváření imunosupresivních faktorů (IL-10, TGF-β, galectin-1, PGE2), aktivace proapoptotických signálních drah v efektorových imunokompetentních buňkách (FasL, TRAIL, IDO, RCAS1), inhibice přímého působení NK buněk (například uvolněním MICA) a inhibice diferenciace a maturace dendritických buněk (STAT3, VEGF, IL-10, SOCS1, arginása). Navíc jsou zde populace buněk, které se podílejí na inhibici imunitní odpovědi, zejména Treg, Tr1 buňky, IL-13 produkující NKT buňky, TAM.

Regulační T lymfocyty

Regulace imunitního systému, zahrnující i protinádorovou imunitní odpověď, patří k základním mechanizmům zachování homeostázy. Imunitní systém je regulován na několika úrovních s cílem udržet v rovnováze odpověď proti antigenům cizím i vlastním. Definování základních principů imunitní odpovědi a její regulace je nezbytné pro správné nastavení léčebné strategie u celé řady různých chorob. Mnoho zásadních poznatků o funkcích a regulacích imunitního systému bylo získáno až v průběhu posledních dvaceti let.

Koncept tzv. „periferní tolerance“ byl ustanoven na podkladě transplantačních a autoimunitních zvířecích modelů, kdy bylo možné přenosem T lymfocytů z “tolerantní” myši zajistit „neodpovídavost“ u „naivního“ příjemce. Subpopulace T lymfocytů, která potlačuje aktivaci a proliferaci efektorových T buněk je známa jako regulační T lymfocyty (Treg). Nejlépe definovaná je podskupina $CD4^+$ T lymfocytů - $CD4^+CD25^+Foxp3^+$, ale jsou popsány i $CD8^+$ buňky s regulační funkcí [29, 134-136].

V minulosti bylo na existenci Treg nahlíženo se střídavým skepticismem, zejména díky nepřítomnosti antigenů, které by je jasně definovaly. Tento fakt změnily až práce Sakaguchiho v polovině 90. let, kdy se regulace imunitní odpovědi a regulační T buňky jako takové dostaly na výsluní zájmu imunologů [137-140].

Současný pohled

Dnes víme, že Treg jsou součástí normálně fungujícího imunitního systému, kdy mají zásadní roli při navození tolerance vůči vlastním antigenům. Absence dostatečného počtu Treg vede na straně jedné k těžkým autoimunitním onemocněním, na straně druhé neadekvátní množství či působení Treg negativně ovlivňuje imunitní odpověď vůči nádorovým buňkám (mylně považovaných za buňky vlastní) a přispívá tak k rozvoji nádorového onemocnění.

Jako negativní prognostický parametr u některých typů malignit bylo popsáno jak zvýšené zastoupení Treg v periferní cirkulaci pacientů, tak zejména jejich akumulace v nádorovém mikroprostředí [32, 141, 142]. Tento vztah byl prokázán i u dlaždicobuněčných karcinomů v oblasti hlavy a krku (HNSCC) [34, 143-145].

Doposud byly popsány minimálně tři základní typy lidských regulačních $CD4^+$ T buněk. První skupina je definována jako $CD4^+CD25^+IL\text{-}10^+Foxp3^{low}$ buňky a označuje se jako regulační buňky 1. typu. Jejich aktivace probíhá při setkání s antigenem a je závislá na IL-10 [146]. Druhou skupinu tvoří přirozeně vznikající regulační T lymfocyty ($CD4^+CD25^{high}Foxp3^+$ T cells (Treg), které vyzrávají ve specializovaném mikroprostředí thymu a mají schopnost inhibovat jak $CD4^+CD25^-$ tak i $CD8^+CD25^-$ T buňky. Mohou působit jak přímým kontaktem buňky regulační a buňky cílové, tak i prostřednictvím solubilních cytokinů (IL-10, TGF- β, IL-35). Většina autorit se v dnešní době shoduje, že mezibuněčný kontakt či alespoň přiblížení je nutné a působení prostřednictvím solubilní cytokinů se uplatňuje parakrinně či v okamžiku mezibuněčného kontaktu [139, 147]. Třetí skupinu tvoří Th3 lymfocyty, které jsou antigen-specifické a produkují ve vysokých koncentracích cytokiny s imunosupresivním efektem (TGF-β a IL-10) [148].

Znaky definující Treg

Vyzrávání regulačních T lymfocytů v thymu i jejich setrvávání v periferní cirkulaci je závislé na IL-2, samy o sobě však nejsou schopné IL-2 produkovat. Receptor pro IL-2 se skládá z tří částí - alfa podjednotky (IL-2Rα, neboli CD25), která podmiňuje vysokou afinitu vazby cytokinu na receptor, beta podjednotky (IL-2Rβ, neboli CD122) a společné podjednotky gama (γc neboli CD132). Vazbou IL-2 na alfa podjednotku receptoru dojde k aktivaci signalizačních kaskád STAT5, MAPK a PI3K [134].

CD25 receptor není unikátní pouze pro regulační T lymfocyty, ale je ve zvýšené míře exprimován na efektorových T lymfocytech po aktivaci jejich TCR (receptor T buněk rozpoznávající antigen). Nicméně populace $CD4^+CD25^+$ T lymfocytů se skládají zejména z buněk s inhibiční aktivitou.

Klíčovou úlohu pro Treg během vývoje v thymu a pro regulaci jejich funkce hraje transkripční faktor „forkhead box P3“ (Foxp3). Bývá také označován jako „master regulator“. Foxp3 moduluje rozdílnou odpověď na stimulaci TCR u regulačních a u efektorových T lymfocytů. Gen pro Foxp3 je lokalizován na X chromozómu. Samotný transkripční faktor Foxp3 má tři základní funkční domény, které ovlivňují celou řadu biologických procesů. Foxp3 pozitivita je vysoce specifická pro $CD4^+25^+$ thymocyty a pro myší periferní T regulační buňky. U lidí je exprimován Foxp3 i na jiných než periferních regulačních T lymfocytech včetně nádorových buněk [149], což zároveň s intracelulární lokalizací tohoto znaku limituje jeho použitelnost pro izolaci a studium subpopulace regulačních buněk u pacientů [138-140, 150].

Na povrchu Treg dále nacházíme celou řadu dalších znaků zodpovědných za inhibiční a modulační signály, nejvíce prostudované jsou molekuly CD28, CTLA-4 a GITR. CD28 (všeobecně známý kostimulační receptor na konvenčních T lymfocytech) je zároveň nezbytný pro vývoj T reg v thymu, kde CD28 kostimulace vyvíjejících se thymocytů indukuje expresi Foxp3, tedy nezávisle na produkci IL-2 [150, 151].

CTLA-4 (Cytotoxic T-Lymphocyte-Associated Antigen 4) je tzv. Counter-receptor pro skupinu B7 kostimulačních molekul (CD80, CD86) antigen prezentujících buněk (APC). Na rozdíl od CD28, působí CTLA-4 při vazbě na CD80,CD86 inhibičně a indukuje produkci indolamin-2,3-dioxygenázy (IDO). Výsledkem je katabolismus tryptofanu, jeho lokální deplece a negativní regulace imunitní odpovědi [152].

GITR (Glucocorticoid-Induced Tumour-Necrosis Factor Receptor Related Protein) je exprimován na povrchu Treg, ale je také v různé míře přítomen na efektorových T buňkách a APCs. Zatím není znám přesný mechanizmus jeho inhibičního působení, ale může negativně ovlivnit aktivaci buněk [137].

Dalším povrchovým znakem, využitelným pro detekci Treg je molekula CD127, α-řetězec receptoru pro IL-7, kdy Treg jsou $CD127^{low}$ populace a konvenční T buňky jsou $CD127^{high}$. Povrchová exprese CD127 zároveň negativně koreluje s Foxp3. CD127 se zdá být využitelná pro průkaz lidských Treg pro funkční studie a potenciální terapeutické využití [153].

Mechanizmus působení

Přesný a univerzální mechanizmus působení Treg je klíčovou otázkou, na kterou ale prozatím neexistuje jednoznačná odpověď. Existují dvě hlavní možné varianty: působení prostřednictvím sekrece inhibičních cytokinů a přímé působení kontaktem mezi efektorovou a supresorovou buňkou. Treg mohou také působit nepřímo, prostřednictvím ovlivnění dendritických buněk [139].

Do první skupiny se řadí hlavní inhibiční cytokiny - IL-10 a TGF-β. Jejich imunosupresní efekt byl mnohokrát prokázán v *in vitro* experimentech a i v mnoha *in vivo* modelech je nezpochybnitelný [154, 155]. V poslední době je zdůrazňován význam dalšího cytokinu s inhibiční aktivitou – interleukinu 35 (IL-35) [139].

V případě Treg a ovlivnění efektivní protinádorové odpovědi je možné nalézt velké množství prací, zdůrazňujících význam kontaktu mezi buňkou regulační a efektorovou [156-158]. Cytolytický mechanizmus při přímém kontaktu buněk je zprostředkován zejména interakcí granzymu A, B a perforinu s molekulou CD18

[159]. Tento mechanizmus byl prokázán při ovlivnění funkce jak B lymfocytů, tak i NK buněk a cytotoxických T lymfocytů [160, 161].

Asi největší pozornost je v poslední době věnována mechanizmu takzvané metabolické disrupce. Tento pojem zahrnuje konzumpci IL-2. Treg exprimují trimerickou formu receptoru pro IL-2 (podjednotky alfa, beta a gama, podrobně vysvětleno výše), ke které má IL-2 mnohonásobně (až 1000x) vyšší afinitu, což vede k jeho konzumpci, a tím snížení lokální dostupnosti pro dělící se efektorové buňky [162]. Dvě práce publikované v roce 2007 prokázaly význam adenosinu pro funkci T regulačních lymfocytů. Bopp a spol. [163] dokládá, že pro supresní funkci Treg je nutný mezibuněčný kontakt prostřednictvím „gap junction“ a transmembránového přenosu cAMP (cyklický adenosin monofosfát) do cílové T buňky [163]. Deaglio a spol. [164] naopak předpokládají význam dvou povrchově vázaných enzymů – CD73 – ecto-5-nukleotidázy, která je exprimovaná zároveň s CD39 – exonukleosid trifosfát difosfohydrolázou-1, které z extracelulárních nukleotidů produkují v bezprostředním okolí buňky vysokou koncentraci adenosinu. Adenosin se naváže na inhibiční A2A receptor aktivovaných efektorových T buněk [164].

Posledním možným mechanizmem ovlivnění průběhu imunitní reakce je vzájemné ovlivňování Treg a plasmocytoidních dendritických buněk (pDCs) a dalších antigen prezentujících buněk, které jsou nezbytné pro aktivaci efektivní T lymfocytární odpovědi [17, 165]. V tomto případě působí Treg inhibičně prostřednictvím vazby CTLA-4 na ko-stimulační molekuly CD80 nebo CD86 a produkcí indolamin-2,3-dioxygenázy (IDO). Tento enzym zvyšuje množství katabolitů tryptofanu, které opět inhibičně ovlivňují efektorové T buňky prostřednictvím pro-apoptotických signálů [166, 167].

Význam

V minulosti probíhající studie byly limitovány nedostatkem znalostí o specifických povrchových znacích a nemožností definovat přesný mechanizmus působení Treg. V současné době je známo množství povrchových i intracelulárních markerů, jejichž kombinace vede k velmi přesné selekci populace T lymfocytů s inhibičními účinky. To umožnilo poodhalení významu Treg v patofyziologii mnohých onemocnění.

Bylo prokázáno, že pro vývoj Treg v thymu a pro expresi Foxp3 je klíčová signalizace zprostředkovaná transformačním růstovým faktorem beta (TGF-β) [168] a zároveň, že TGF-β ovlivňuje Treg v průběhu kojení a tím hraje zásadní roli při navození tolerance proti antigenům obsaženým v mateřském mléce. Tento fakt potvrzuje zásadní význam kojení pro prevenci vzniku budoucího alergického onemocnění [169].

V oblasti protinádorové imunologie bylo prokázáno, že vzestup hladiny Treg negativně ovlivňuje efektivní imunitní reakci proti nádorovým buňkám a v řadě případů koreluje s horší prognózou onkologicky nemocného pacienta [149]. Zároveň bylo prokázáno, že Treg jsou hlavní překážkou pro imunoterapii a aktivní vakcinaci [170].

Malignancy	*N pts*	*Prognostic value*
Breast carcinoma (Bates *et al.*, 2006)	237	Unfavorable
Breast carcinoma (Gobert *et al.*, 2009)	191	Unfavorable
Colorectal carcinoma (Salama *et al.*, 2009)	967	Favorable
Cervix carcinoma (Jordanova *et al.*, 2008)	115	Unfavorable
Esophagus carcinoma (Yoshioka *et al.*, 2008)	122	Not significant
Gastric carcinoma (Mizukami *et al.*, 2008)	80	Unfavorable (*)
Head & neck carcinoma (Badoual *et al.*, 2006)	84	Favorable
Hepatocellular carcinoma (Gao *et al.*, 2007)	302	Unfavorable
Kidney carcinoma (Siddiqui *et al.*, 2007)	170	Not significant
Lymphoma (B cell) (Carreras *et al.*, 2006)	98	Favorable
Lymphoma (Hodgkin) (Alvaro *et al.*, 2005)	257	Favorable
Ovary carcinoma (Curiel *et al.*, 2004)	104	Unfavorable
Pancreatic carcinoma (Hiraoka *et al.*, 2006)	198	Unfavorable

Obrázek 7 – Prognostická role Treg v různých typech malignit. Převzato z Martin, 2010 [149].

Paradoxní role Treg

S rostoucím objemem informací o mechanizmech protinádorové imunitní odpovědi a zejména o jejím možném pozitivním i negativním ovlivnění se pochopitelně zvyšuje i množství poznatků, které ještě přesně pochopit nedokážeme anebo pro ně hledáme vysvětlení jen obtížně. Jednou z těchto doposud nezodpovězených otázek je, v některých pracích uváděná, korelace vysoké hladiny Treg s pozitivní prognózou či jejich nulová prognostická hodnota. V souladu s předchozími výsledky preklinických studií [171, 172], shrnul Martin [149] význam Treg u různých typů lidských nádorů (viz. Obrázek 1). 7 z celkově 13 prací hodnotících význam Treg udává negativní prognostickou hodnotu (vysoké hladiny Treg korelují s negativní prognózou). Naopak u 4 korelovala zvýšená hodnota Treg s lepší prognózou a u dvou prací nenašli autoři žádnou statistickou významnost. Je nutné zdůraznit, že tento paradoxní jev je popsán již na řadě různých malignit, včetně solidních nádorů. Salama a spol. publikovali práci, kde byla u pacientů s kolorektálním karcinomem lepší prognóza asociovaná s vyšší denzitou intratumorálních Foxp3+ Treg [173]. Autoři tato data označili za překvapivá, neočekávaná a v přímém rozporu s dosavadním názorem, že přítomnost Treg v nádorovém mikroprostředí je negativním prognostickým znakem [32, 174, 175]. U pacientů s folikulárním lymfomem a Hodginským lymfomem je dnes jasně prokázáno, že vysoký počet infiltrujících Treg je asociován s delším časem do progrese onemocnění i s delším celkovým přežitím [176-178]. Obdobná data, prokazující pozitivní korelaci vysokého zastoupení Treg s prognózou pacientů byla prokázána i u pacientů s nádory hlavy a krku, kde vysoké zastoupení Foxp3+ Treg bylo asociováno s lepší lokoregionální kontrolou nádorů [179]. Tato práce prokázala, že jedinými dvěma statisticky významnými faktory pro lokoregionální kontrolu HNSCC bylo T stádium onemocnění a Treg infiltrace v nádoru. Po ukončení onkologické léčby byla také frekvence Foxp3+ CD4+ Treg v periferní krvi vyšší u pacientů v remisi než u pacientů s recidivou [143].

Jaké je ale vysvětlení paradoxního „pozitivního“ působení Treg?

Tyto zdánlivě rozporuplné výsledky o významu Treg u pacientů s nádorovým onemocněním lze vysvětlit řadou mechanizmů. V první řadě se zdá, že význam Treg je různý v závislosti na stádiu onemocnění. Například pacienti v časném stádiu (1. stádium) nemalobuněčného karcinomu plic a s vysokým zastoupením Treg mezi nádor infiltrujícími lymfocyty mají statisticky významně vyšší pravděpodobnost recidivy onemocnění [180]. U pacientů s ovariálním karcinomem je bez závislosti na pokročilosti onemocnění zastoupení Treg asociováno s horší prognózou, ovšem u pacientů s pokročilým stádiem onemocnění nebo v případě metastatického postižení je absolutní počet nádor infiltrujících Foxp3+ Treg naopak nezávislým faktorem pro delší přežití vztažené na samotné nádorové onemocnění (disease-specific survival) [181]. Tato data jsou v souladu s preklinickými studiemi na myších, kdy Treg jsou často označovány za hlavní mechanizmus, kterým nádory v časné fázi svého růstu dokáží obejít účinnou protinádorovou odpověď. Eliminace Treg před setkáním s nádorovými buňkami (před inokulací nádoru) vede u většiny pokusných zvířat k rozvoji efektivní protinádorové imunitní odpovědi a dlouhodobému přežívání bez vzniku nádorového onemocnění. Naopak eliminace Treg u myší s rozvinutým nádorovým onemocněním nemá žádný terapeutický efekt [182, 183].

Druhé vysvětlení vychází z faktu, že negativní efekt Tregs je zprostředkován zejména jejich inhibičním působením na protinádorové efektorové lymfocyty. Proto různé studie uvádějí jako mnohem důležitější parametr pro přežití pacientů poměr mezi CD8 T buňkami a Treg než hodnotu samotných Treg či hodnotu samotných infiltrujících CD8 buněk [184, 185]. Hodnocení pouze hladin samotných Treg, bez znalosti poměrů mezi Treg a efektorovými buňkami, může vést k některým nepřesnostem v interpretaci výsledků.

V neposlední řadě, na rozdíl do myších modelů, Foxp3 znak, který je často používán pro definování Treg, není u lidí exprimován pouze touto buněčnou populací. Bylo prokázáno, že Foxp3 je přechodně exprimován i aktivovanými CD4+ CD25+ efektorovým T lymfocyty, které nevykazují žádnou inhibiční aktivitu [186, 187]. Dále je Foxp3 exprimován i podskupinou CD8+ lymfocytů (dnes velmi

aktuální a zajímavá skupina regulačních CD8+ buněk) a i samotnými nádorovými buňkami [149, 188]. Ačkoliv je Foxp3 zatím nejlepším markerem pro identifikaci Treg, použití pouze tohoto markeru může vést k nadhodnocení zastoupení této subpopulace. V různých studiích se liší poměr aktivovaných CD4 nebo CD8 buněk exprimujících Foxp3+ infiltrujících nádorové tkáně a vyčíslení pouze Foxp3+ buněk bez funkčních analýz nemusí odpovídat skutečnému počtu regulačních T buněk a může vést k mylné interpretaci výsledků.

Pozitivní roli Treg v posílení protinádorové imunity a tím ovlivnění prognózy pacientů s nádorovým onemocněním je dále možné vysvětlit dvěma obecnými hypotézami. U hematologických malignit a u některých solidních nádorů (HNSCC, kolorektální karcinom), kde vysoké zastoupení Treg koreluje s pozitivní prognózou, je nádorová tkáň zároveň silně infiltrována i buňkami přirozené imunity (makrofágy, neutrofily) produkujícími prozánětlivé cytokiny, růstové faktory a pro-angiogenní molekuly stimulující neoangiogenezi a nádorový růst. Pokud Treg sníží dostupnost takto „pronádorově“ působících cytokinů a růstových faktorů, může to zpomalit růst nádoru [189, 190]. Experimentálně byla na myším modelu tato schopnosti Treg potlačit zánětlivou odpověď iniciovanou mechanizmy přirozené buněčné imunity prokázána [191]. U lidí mohou Treg exprimující FasL, granzym či perforin indukovat apoptózu u monocytů a makrofágů a tím snížit jejich pronádorové působení v nádorovém mikroprostředí [159, 192, 193]. Rovněž bylo prokázáno, že adoptivní přenos CD4+CD25+ Treg může velmi rychle zpomalit růst kolorektálních karcinomů indukcí apoptózy, což naznačuje, že Treg přímo či nepřímo spouští apoptózu samotných nádorových buněk [171].

Jak se ukazuje, pochopení složitostí nádorového růstu, zejména interakcí v nádorovém prostředí, kde se vlivem celé řady buněk uplatňují různé regulační mechanizmy, jsme ještě dosti vzdáleni. Pokud porozumíme skutečnému mechanizmu inhibičního působení regulačních T buněk, i v celé šíři souvislostí s nádorovým mikroprostředím, zejména ve vztahu k samotným nádorovým

buňkám, k podpůrným buňkám a k dalším imunokompetentním buňkám [194-200], pak můžeme uvažovat o zlepšení dosavadních terapeutických výsledků.

Teorie nádorové kmenové buňky u HNSCC

V posledních přibližně dvaceti letech byla u hematoonkologických malignit (experimentálně poprvé Johnem Dickem v roce 1994) a poté i u solidních nádorů (poprvé v roce 2003, Michael Clark, u nádoru prsu) identifikovaná skupina buněk, označovaných jako kmenové nádorové buňky (cancer stem cells, CSCs), které jsou dávány do souvislosti se vznikem a chováním nádoru [201-203]. Dle této teorie je biologická povaha nádorových buněk, včetně jejich schopnosti metastazovat, podmíněna vlastnostmi a chováním kmenových nádorových buněk [204]. Tyto buňky mají schopnost sebeobnovy, iniciace a potenciace nádorového růstu, diseminace z tkáně tumoru do krevního řečiště a zároveň i schopnosti z řečiště vystoupit a proniknout do tkáně na jiném místě organizmu, tedy metastazovat [205, 206]. Metastatický potenciál je jistě potencován i schopností kmenové nádorové buňky produkovat fenotypicky i funkčně různorodé nádorové buňky [203]. Navíc jsou CSCs vybaveny řadou mechanizmů, které je činní rezistentními k chemoterapeutikům a tím znemožňují kompletní eliminaci nádorového onemocnění. Porozumění biologickým mechanizmům CSCs a zacílení léčby na jejich jednotlivé molekulární mechanizmy by bylo klíčovým krokem k nalezení efektivní léčby [207, 208]. Markery kmenových buněk solidních nádorů jsou v celé řadě případů shodné se znaky hematopoetických kmenových buněk jako jsou CD44+CD24-low, CD133+, CD29+, CD28+, CD166+ [209]. Side population (SP) je podskupina buněk (běžně 0,1% až 10% buněk), která vykazuje řadu vlastností shodných jako buňky kmenové a je vyselektována na základě specifických fluorescenčních vlastností podmíněných schopností vylučovat Hoechst 33342 [210, 211]. SP byla izolována u řady solidních nádorů. Je tvořena buněčnou populací vykazující vysoké zastoupení znaků asociovaných s kmenovými buňkami i řadu dalších vlastností, jako je schopnost sebeobnovy a schopnost vzniku řady různě diferencovaných buněčných typů [210, 212]. Hoechst 33342 je u SP vylučován díky expresi ATP vázajících membránových transportních proteinů (ABC) [209]. Nejznámější je ABC B1, neboli p-glykoprotein, v poslední době je jako nejvýznamnější ale označován ABC G2 [209]. Odolnost SP proti konvenčním chemoterapeutikům je zprostředkována zvýšenou expresí ABC G2 a transportéry ABC B1, ABC C1 a ABC G2, které tvoří

skupinu tzv.multidrug-resistance genů [213, 214]. Navíc buňky SP vykazují řadu vlastností podobných progenitorovým buňkám, což výrazně zvyšuje pravděpodobnost nádorového růstu při aplikaci pokusným zvířatům ve srovnání s non-SP populací [212, 215-217]. Opakovaně bylo prokázáno, že nádory a buněčné linie s vysokým metastatickým potenciálem obsahují vysoké procento SP populace. Bylo prokázáno, že vyselektovaná SP populace je rezistentní například k Bortezomibu či etoposidu, což bylo v souladu s vysokým zastoupením ABC G2 transportéru [218, 219]. Navíc u SP z vysoce invazivních nádorů byla prokázaná abnormální aktivita Wnt signalizační dráhy [220]. Tyto informace naznačují, že SP může být u HNSCC, obdobně jako pro jiné malignity, hlavní buněčnou podskupinou zodpovědnou za nádorový růst a metastazování. Wnt signalizační cesta by mohla být zajímavým terapeutickým cílem, její inhibice by měla vést k eliminaci nádorových kmenových buněk a tím k eliminaci HNSCC [221].

Table 1 | **ABC transporters involved in drug resistance**

Gene	Protein/alias	Chemotherapeutic drugs effluxed by transporter	Other drugs and substrates
ABCA2	ABCA2	Estramustine	–
ABCB1	PGP/MDR1	Colchicine, doxorubicin, etoposide, vinblastine, paclitaxel	Digoxin, saquinivir,
ABCC1	MRP1	Doxorubicin, daunorubicin, vincristine, etoposide, colchicine, camptothecins, methotrexate	Rhodamine
ABCC2	MRP2	Vinblastine, cisplatin, doxorubicin, methotrexate	Sulfinpyrazone
ABCC3	MRP3	Methotrexate, etoposide	–
ABCC4	MRP4	6-mercaptopurine, 6-thioguanine and metabolites; methotrexate	PMEA, cAMP, cGMP
ABCC5	MRP5	6-mercaptopurine, 6-thioguanine and metabolites	PMEA, cAMP, cGMP
ABCC6	MRP6	Etoposide	–
ABCC11	MRP8	5-fluorouracil	PMEA, cAMP, cGMP
ABCG2	MXR/BCRP	Mitoxantrone, topotecan, doxorubicin, daunorubicin, irinotecan, imatinib, methotrexate	Pheophorbide A, Hoechst 33342, rhodamine

ABC, ATP-binding cassette; BCRP, breast cancer resistance protein; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanine monophosphate; MDR, multidrug resistance; MRP, multidrug-resistance-associated protein; MXR, mitoxantrone resistance protein; PMEA, 9-[2-(phosphonomethoxy)ethyl]adenine.

Obrázek 8 - Rodina ABC transportních proteinů a terapeutika, která tyto transportéry přenášejí. Převzato z Dean, 2005 [209].

Pokroky v systémové léčbě nádorů hlavy a krku

Recidivující či metastatické spinocelulární karcinomy v oblasti hlavy a krku jsou onemocnění s velmi nepříznivou prognózou, medián přežití se pohybuje okolo 6 měsíců [222]. Celková odpověď na léčbu v případě užití jednoho typu systémového léčiva se pohybuje mezi 15 a 30 %. O něco vyšší účinnosti je dosaženo kombinací léčiv, bohužel celkové přežití těchto pacientů se zatím nepodařilo prodloužit [223]. Zlepšení tohoto nepříznivého stavu je cílem celé řady odborných týmů, využívajících jak změn v dávkovacích režimech či kombinacích léčiv, tak jejich chemických modifikací či úplně nových typů léčiv.

Řada studií přinesla povzbudivé dílčí výsledky, zde uvedeme ty nejvýznamnější z poslední doby, které ovlivnily oblast onkologie hlavy a krku.

Studie EXTREME (Erbitux in first-line Treatment for Reccurrent or Metastatic Head and Neck Cancer) [224] prokázala povzbudivé výsledky na 442 pacientech léčených buď cisplatinou nebo karboplatinou (100 mg/m2) následovanou 5-FU (1000 mg/m2/den, 4 dny každé 3 týdny, minimálně 6x) anebo tím samým schématem následovaným podáním cetuximabu (monoklonální protilátka proti receptoru pro epidermální růstový faktor, v iniciální dávce 400 mg/m2, následovanou 250mg/m2 týdně). Celkové průměrné přežití bylo přidáním cetuximabu prodlouženo ze 7,4 měsíců na 10,1 měsíce (p= 0,004), se srovnatelným procentem vedlejších nežádoucích účinků. Studie mimo jiné prokázala, že počet kopií genu *EGFR* (detekováno pomocí FISH) nepredikuje účinnost cetuximabu.

Obdobné schéma měla i studie používající místo cetuximabu panitumumab (plně humanizovanou monoklonální protilátku proti EGFR), jejíž výsledky by měly být dostupné v tomto roce [222].

Výsledky další slibné studie byly publikovány Airoldim [225], který použil kombinaci paclitaxelu (80 mg/m^2 týdně) a pegylovaného liposomálního doxorubicinu - Caelyxu (12,5 mg/m^2 dvakrát týdně). Celkové přežití bylo v této studii 10 měsíců a u 73% pacientů bylo dosaženo pozitivní odpovědi na léčbu (disease control rate).

Liposomální forma léčiva se osvědčila i v randomizované multicentrické studii (fáze III) vedenou Jehnem [226]. Autoři v klasické kombinaci cisplatina/5-FU použili liposomální formu cisplatiny – lipoplatinu. V obou ramenech studie bylo ve stejném procentu dosaženo kontroly onemocnění, zásadní výhoda lipoplatiny však byla v nižším výskytu nežádoucích účinků, zejména nefrotoxicity.

Na rozdíl od cetuximabu bylo zatím neuspokojivých výsledků u nádorů hlavy a krku dosaženo kombinací stávajících standardních terapeutických režimů s tyrosin kinázovými inhibitory, které jsou již dnes běžně využívány v klinické praxi v jiných indikacích. Ve studie IMEX bylo randomizováno 482 pacientů do 3 ramen, EGFR tyrozin kinázový inhibitor gefitinib v dávkování 250 mg/d a 500 mg/d ve srovnání s metotrexátem 40 mg/m^2. Průměrné přežití bylo pouze 5,6 resp. 6 resp. 6,7 měsíce. Obdobně neuspokojivých výsledků bylo zatím dosaženo i při použití další tyrosin-kinázových inhibitorů jako sunitinibu, erlitinibu či lapatinibu [227]. Jediným nadějným výsledkem byla studie ECOG [222], kdy kombinace docetaxelu (35 mg/m^2) a gefitinibu (250 mg/d) posunula čas do progrese (time to progression) z 2 na 3,5 měsíce, neměla však vliv na celkové přežití.

Z dalších, měně známých prostředků biologické léčby je možné zmínit 3-AP Triapine (3-aminopyridin-2-carboxaldehyd thiosemicarbazon), což je inhibitor ribonukleotid reduktázy. V multicentrické studii fáze II [228] bylo dosaženo celkové odpovědi 5,9% a čas do progrese onemocnění byl 3,9 měsíce. Lonafarnib je specifický inhibitor farnesyl transferázy, jeho užití ve studii fáze II u pacientů refrakterních na platinu prokázalo průměrný čas do progrese 2,04

měsíce a celkové přežití 9,17 měsíce [229]. Zatím s rozporuplnými výsledky byly i zkoušeny inhibitor src-kináz (Dasatinib) [222] a perorální inhibitor histon-deacetylázy (Vorinostat) [230].

Obdobně jako v jiných oblastech onkologie i léčba recidivujících či metastazujících spinocelulárních karcinomů v oblasti hlavy a krku je stále častěji kombinována s imunoterapií. V poslední době nejlepších výsledků (celkové přežití 21,8 měsíce) bylo dosaženo u pacientů s pozitivní odpovědí na léčbu kombinací docetaxel, ifosfamid, cisplatina u nichž následovalo subkutánní podávání nízkých dávek interleukinu-2 (IL-2) a perorálního podávání vitamínu A [231, 232].

Kombinace biologické léčby, směrované léčby a imunoterapie bylo využito v klinické fázi I, kdy byl podáván imunokonjugát Bivatuzumab mertansin, kombinace monoklonální protilátky anti-CD44v6 (bivatuzumab) a mertansinu, což je vysoce účinný blokátor miktrotubulů [233]. Přestože byla tato studie později zastavena pro nežádoucí účinky v podobě kožní toxicity, byla tato studie zajímavá ze dvou hledisek. Zaprvé klinický efekt imunokomplexů koreloval s narůstajícím počtem lymfocytů a NK buněk a poklesem VEGF v nádorové tkáni a zadruhé molekula CD44 je u nádorů hlavy a krku považována za jeden ze znaků buněk kmenových či kmenovým buňkám blízkých (cancer stem like cells, viz.kapitola „Teorie nádorové kmenové buňky u HNSCC“) a tato studie byla vlastně jedna z prvních zamířených přímo proti této buněčné populaci, kterou řada autorů činní zodpovědnou za recidivu a metastatické šíření nádoru a tím selhání léčby.

Směrovaná terapie a polymerní léčiva v léčbě HNSCC

Jak bylo již krátce uvedeno v předchozí kapitole, uplatňují se v moderní chemoterapii jak léčiva úplně nová, tak nové kombinace léčiv či jejich chemické modifikace, které významně ovlivňují jejich vlastnosti a terapeutické výsledky. Dlouhodobé klinické výsledky léčby HNSCC jsou zatím neuspokojivé, proto se nalezení nových struktur, přístupů a strategií jeví jako klíčové [196, 234-236].

Jeden z takových přístupů, jak prolomit tuto hranici je bezpochyby směrovaná terapie. Tento koncept není nový, již na začátku dvacátého století (1905) byly hlavní myšlenky vysloveny Paulem Ehrlichem, který předpověděl léčbu pomocí takzvaných “magic bullet”, tedy léčiva obsahujícího vedle samotné účinné látky i nějakou směrující strukturu [237]. Reálný začátek této éry nastal ale až za téměř 100 let později, kdy do hry vstoupila takzvaná biologická léčba. Imatinib mesylat (Glivec), což je malá molekula nhibující mutovanou tyrosin kinázu BCR-ABL, byla uvedena do klinické praxe [238, 239].

V současné době je s úspěchem využívána řada biologických léčiv, jedním z nejznámějších a nejúspěšnějších je terapie směrovaná proti receptoru pro epidermální růstový faktor (EGFR), ať již v podobě monoklonální protilátky (Erbitux, cetuximab) či v podobě opět malé molekuly inhibující tyrosin-kinázovou aktivitu (Gefitinib – Iressa). Epidermální růstový faktor (EGF) byl objeven v roce 1962 a jeho receptor byl vyizolován a poprvé charakterizován Cohenem v roce 1980 [240, 241].

Americká FDA povolila inhibitor tyrosin-kinázové aktivity EGFR a první monoklonální protilátku směrovanou proti EGFR před téměř 25 lety [242, 243]. Cetuximab (Erbitux), volná monoklonální humanizovaná protilátka proti EGFR je v dnešní době užívána v každodenní klinické praxi v kombinaci s radioterapií pro léčbu lokálně pokročilých spinocelulárních karcinomů v oblasti hlavy a krku [222, 244-250].

Pro budoucí klinické využití bylo připraveno kombinované léčivo, které na bázi HPMA (*N*-(2-hydroxypropyl)methacrylamid) kopolymeru váže nízkomolekulární cytostatikum (doxorubicin) a je směrováno navázanou monoklonální protilátkou proti EGFR.

Koncept využití polymerů jako nosičů pro makromolekulární konstrukt pochází od Ringsdorfa z poloviny 70.tých let [50].

Ve vodě rozpustné polymery na bázi HPMA byly testovány jako výhodné nosiče pro cílenou léčbu. Poly-(HPMA) polymer byl původně připraven Kopečkem [251] začátkem 70.tých let jako plasma expander (Duxon) a v preklinických testech byl shledán netoxickým, neimunogenním a nevázal proteiny krevní plasmy [252].

Opakovaně bylo prokázáno, že vazbou na polymer je snížena imunogenicita vázaného proteinu, je zvýšena jeho účinnost, biokompatibilita a rozpustnost [57, 253, 254]. Lepší farmakokinetické a farmakodynamické vlastnosti, delší cirkulace v krevním řečišti a snížení nespecifické toxicity oproti volnému doxorubicinu či jinému cytostatiku jsou také popsány po jejich navázání na HPMA nosič [253, 255-258]. K vazbě cytostatika je využíván přesně definovaný typ chemické vazby. Jedná se buď o amidovou proteolytickou vazbu anebo o hydrazonovou hydrolytickou vazbu. V prvním případě je prostřednictvím postranních oligopeptidových řetězců vázáno cytostatikum (nejčastěji doxorubicin) a eventuelně i směrující struktury. Složení postranního řetězce je klíčové pro vlastnosti celé makromolekuly a zajišťuje její stabilitu a inaktivitu při transportu v krevním řečišti. Teprve po rozštípnutí postranního řetězce intracelulárními katepsiny (B, D, L, H) dojde k uvolnění vázané aktivní molekuly. V řadě experimentů se jako nejvýhodnější ukázaly vlastnosti tetrapeptidu glycyl-fenylalanyl-leucyl-glycyl (Gly-Phe-Leu-Gly) [259, 260]. V druhém případě je cytostatikum vázáno pomocí hydrolytické hydrazonové vazby, která je velmi

stabilní při pH 7,4, ale při poklesu pH k hodnotám kolem 5 je rychle hydrolyticky štěpena [261]. Tato vlastnost umožní uvolnění léčiva až v endozomech (lyzozomech) buňky, nikoliv předčasně v krevním řečišti [262, 263].

Jedna ze zásadních výhod makromolekulárních polymerů je schopnost pasivního i aktivního směrování. Pasivní směrování, fenomén nazývaný EPR efekt (Enhanced Permeability and Retention effect) se uplatňuje pouze u molekul s molekulovou hmotností větší než 40 kDa, nikoliv u většiny běžně užívaných cytostatik. EPR efekt vychází z vlastnosti kapilár, které na základě proangiogenních stimulů rychle narostly v okolí nádorové tkáně a díky méněcennému endotelu je zvýšená jejich prostupnost – permeabilita. Tím se mohou makromolekuly dostávat do mezibuněčného prostoru, odkud nejsou drénovány díky nepřítomnosti odpovídající sítě lymfatických cév a tak se hromadí v nádorové tkáni [264-266].

Aktivní směrování je umožněno díky vazbě vhodně směrující struktury, která nalezne svůj vazebný bod někde na buněčné membráně nádorové buňky. Jako směrující struktury jsou využívány ligandy (karbohydráty, lektiny, hormony, peptidy) nebo monoklonální protilátky či jejich části. Jako cílová struktura může být využita molekula unikátní pouze pro cílovou buňku anebo molekula exprimovaná na cílové buňce ve velkém množství, řádově vyšším než v okolních tkáních. [267-270].

Další velmi významnou vlastností polymerních konjugátů, krom jejich schopnosti potlačovat růst nádorových buněk, je jejich schopnost v rámci této léčby umožnit vznik efektivní specifické protinádorové imunitní odpovědi a tím výrazně navýšit terapeutický efekt [263, 271-273].

Seznam zkratek

HNSCC - Head and Neck Squamous Cell Carcinoma

EGFR – Epidermal growth factor receptor

KRAS/HRAS – dva z proteinů *Ras*, což jsou malé GTPázy, které se podílejí na buněčné signalizaci vedoucí k růstu, diferenciaci a přežívání

PI3K-AKT – Phosphatidylinositol 3-kináza (PI3Ks) – signalizační molekula podílející se na regulaci buněčné proliferace, přežívání a migrace

FCγRIIa, FCγRIIIa – dva typy Fc gamma receptorů (FCγR) – rozeznávají a vážou Fc fragment protilátek

kinázy Src rodiny – rodina protein-tyrozinázových kináz podílejících se na normálních buněčných funkcích – adhezi, migraci, angiogenezi, přežívání, proliferaci, diferenciaci

MET = HGFR – receptor pro hepatocyte growth factor (HGF)

ERCC1 - excision repair cross-complementation group 1 gene – jeden z genů kódujících proteiny tvořící komplex, který opravuje chemoterapií způsobené poškození DNA (např. cisplatin-induced DNA adducts)

p53 – tumor-supresorový protein uplatňující se v celé řadě buněčných procesů (regulace genové exprese, regulace buněčného růstu, apoptózy, reparace DNA, angiogenezi, procesy v průběhu senecence

p16 – tumor-supresorový protein, inhibitor CDK kináz

MMP9 - Matrix metalloproteináza 9 – zinc-dependent proteináza hrající roli v destrukci a opravě extracelulární matrix a basální membrány v řadě fyziologických i patologických stavů

VEGF – vascular endothelial growth factor

Akt - serine-threonine protein kináza – podílí se angiogenezi, stimuluje buněčný cyklus, diferenciaci a buněčný růst,

FasL – Fas ligand – ligand vázající se na Fas receptor, po navázání spouští apoptotickou kaskádu v cílové buňce, molekulární mechanizmus využíván řadou buněk, například v regulaci buněčné odpovědi, využíván i nádorovými buňkami k uniknutí protinádorové imunitě

IHC - imunohistochemie

Literatura

1. WHO. *WHO - features*. 2010; Available from: http://www.who.int/features/qa/15/en/; http://www.pitt.edu/~super1/lecture/lec0192/014.htm.
2. WHO, *Global health risks: mortality and burden of disease attributable to selected major risks.* 2009.
3. ÚZIS, *Zdravotnická ročenka České republiky.* 2009.
4. Yamamoto, Y., et al., *Occult papillary carcinoma of the thyroid. A study of 408 autopsy cases.* Cancer, 1990. **65**(5): p. 1173-9.
5. Sampson, R.J., et al., *Occult thyroid carcinoma in Olmsted County, Minnesota: prevalence at autopsy compared with that in Hiroshima and Nagasaki, Japan.* Cancer, 1974. **34**(6): p. 2072-6.
6. Harach, H.R., K.O. Franssila, and V.M. Wasenius, *Occult papillary carcinoma of the thyroid. A "normal" finding in Finland. A systematic autopsy study.* Cancer, 1985. **56**(3): p. 531-8.
7. Fukunaga, F.H. and R. Yatani, *Geographic pathology of occult thyroid carcinomas.* Cancer, 1975. **36**(3): p. 1095-9.
8. Kalayjian, R.C., et al., *Age-related immune dysfunction in health and in human immunodeficiency virus (HIV) disease: association of age and HIV infection with naive CD8+ cell depletion, reduced expression of CD28 on CD8+ cells, and reduced thymic volumes.* J Infect Dis, 2003. **187**(12): p. 1924-33.
9. Pittsbourg, U.o. *Cancer epidemiology*. 2010; Available from: http://www.pitt.edu/~super1/lecture/lec0192/014.htm.
10. WHO. *WHO - healthinfo*. 2010; Available from: http://www.who.int/healthinfo/global_burden_disease/global_health_risks/en/index.html, .
11. Psyrri, A., et al., *Head and neck cancer.* J Oncol, 2009. **2009**: p. 358098.
12. Parkin, D.M., et al., *Global cancer statistics, 2002.* CA Cancer J Clin, 2005. **55**(2): p. 74-108.
13. Egloff, A.M. and J.R. Grandis, *Targeting epidermal growth factor receptor and SRC pathways in head and neck cancer.* Semin Oncol, 2008. **35**(3): p. 286-97.
14. Egloff, A.M. and J.R. Grandis, *Improving Response Rates to EGFR-Targeted Therapies for Head and Neck Squamous Cell Carcinoma: Candidate Predictive Biomarkers and Combination Treatment with Src Inhibitors.* J Oncol, 2009. **2009**: p. 896407.
15. Fountzilas, G., et al., *MMP9 but Not EGFR, MET, ERCC1, P16, and P-53 Is Associated with Response to Concomitant Radiotherapy, Cetuximab, and Weekly Cisplatin in Patients with Locally Advanced Head and Neck Cancer.* J Oncol, 2009. **2009**: p. 305908.
16. Ferrari, D., et al., *Biomolecular markers in cancer of the tongue.* J Oncol, 2009. **2009**: p. 412908.
17. Bordon, Y., *Present and clear danger.* Nat Rev Immunol, 2010. **10**(8): p. 616.
18. Simons, A.L., et al., *Inhibition of glutathione and thioredoxin metabolism enhances sensitivity to perifosine in head and neck cancer cells.* J Oncol, 2009. **2009**: p. 519563.
19. Knauer, S.K., *Prognostic and therapeutic potential of nuclear receptors in head and neck squamous cell carcinomas.* J Oncol, 2009. **2009**: p. 349205.
20. Chen, J.S., et al., *EGFR regulates the side population in head and neck squamous cell carcinoma.* Laryngoscope, 2006. **116**(3): p. 401-6.
21. Plzak, J., et al., *Epithelial-stromal interaction in squamous cell epithelium-derived tumors: an important new player in the control of tumor biological properties.* Anticancer Res, 2010. **30**(2): p. 455-62.

22. Strnad, H., et al., *Head and neck squamous cancer stromal fibroblasts produce growth factors influencing phenotype of normal human keratinocytes.* Histochem Cell Biol, 2010. **133**(2): p. 201-11.
23. Campbell, I., W. Qiu, and I. Haviv, *Genetic changes in tumour microenvironments.* J Pathol, 2011. **223**(4): p. 450-8.
24. Allen, M. and J. Louise Jones, *Jekyll and Hyde: the role of the microenvironment on the progression of cancer.* J Pathol, 2010. **223**(2): p. 162-76.
25. Polyak, K., I. Haviv, and I.G. Campbell, *Co-evolution of tumor cells and their microenvironment.* Trends Genet, 2009. **25**(1): p. 30-8.
26. Polyak, K. and W.C. Hahn, *Roots and stems: stem cells in cancer.* Nat Med, 2006. **12**(3): p. 296-300.
27. Boucek, J., et al., *Regulatory T cells and their prognostic value for patients with squamous cell carcinoma of the head and neck.* J Cell Mol Med, 2010. **14**(1-2): p. 426-33.
28. Andl, T., et al., *Etiological involvement of oncogenic human papillomavirus in tonsillar squamous cell carcinomas lacking retinoblastoma cell cycle control.* Cancer Res, 1998. **58**(1): p. 5-13.
29. Nishikawa, H. and S. Sakaguchi, *Regulatory T cells in tumor immunity.* Int J Cancer, 2010. **127**(4): p. 759-67.
30. Mantovani, A. and A. Sica, *Macrophages, innate immunity and cancer: balance, tolerance, and diversity.* Curr Opin Immunol, 2010. **22**(2): p. 231-7.
31. Sica, A., *Macrophages give Gas(6) to cancer.* Blood, 2010. **115**(11): p. 2122-3.
32. Curiel, T.J., et al., *Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival.* Nat Med, 2004. **10**(9): p. 942-9.
33. Liyanage, U.K., et al., *Prevalence of regulatory T cells is increased in peripheral blood and tumor microenvironment of patients with pancreas or breast adenocarcinoma.* J Immunol, 2002. **169**(5): p. 2756-61.
34. Schaefer, C., et al., *Characteristics of CD4+CD25+ regulatory T cells in the peripheral circulation of patients with head and neck cancer.* Br J Cancer, 2005. **92**(5): p. 913-20.
35. Albers, A.E., et al., *Immune responses to p53 in patients with cancer: enrichment in tetramer+ p53 peptide-specific T cells and regulatory T cells at tumor sites.* Cancer Immunol Immunother, 2005. **54**(11): p. 1072-81.
36. Shevach, E.M., *Regulatory/suppressor T cells in health and disease.* Arthritis Rheum, 2004. **50**(9): p. 2721-4.
37. Kuss, I., et al., *Decreased absolute counts of T lymphocyte subsets and their relation to disease in squamous cell carcinoma of the head and neck.* Clin Cancer Res, 2004. **10**(11): p. 3755-62.
38. Kuss, I., et al., *Imbalance in absolute counts of T lymphocyte subsets in patients with head and neck cancer and its relation to disease.* Adv Otorhinolaryngol, 2005. **62**: p. 161-72.
39. Boucek, J., et al., *Regulatory T cells and their prognostic value for patients with squamous cell carcinoma of the head and neck.* Journal of cellular and molecular medicine, 2010. **14**(1-2): p. 426-33.
40. Molling, J.W., et al., *Low levels of circulating invariant natural killer T cells predict poor clinical outcome in patients with head and neck squamous cell carcinoma.* Journal of clinical oncology : official journal of the American Society of Clinical Oncology, 2007. **25**(7): p. 862-8.
41. Dong, H.P., et al., *NK- and B-cell infiltration correlates with worse outcome in metastatic ovarian carcinoma.* Am J Clin Pathol, 2006. **125**(3): p. 451-8.
42. Karcher, J., et al., *Antitumor vaccination in patients with head and neck squamous cell carcinomas with autologous virus-modified tumor cells.* Cancer Res, 2004. **64**(21): p. 8057-61.

43. Janke, M., et al., *Activation of human T cells by a tumor vaccine infected with recombinant Newcastle disease virus producing IL-2.* Int J Oncol, 2008. **33**(4): p. 823-32.
44. Rapidis, A.D. and G.T. Wolf, *Immunotherapy of head and neck cancer: current and future considerations.* J Oncol, 2009. **2009**: p. 346345.
45. Leemans, C.R., B.J. Braakhuis, and R.H. Brakenhoff, *The molecular biology of head and neck cancer.* Nat Rev Cancer, 2011. **11**(1): p. 9-22.
46. Tran, N., B.R. Rose, and C.J. O'Brien, *Role of human papillomavirus in the etiology of head and neck cancer.* Head Neck, 2007. **29**(1): p. 64-70.
47. Wald, A.I., et al., *Alteration of microRNA profiles in squamous cell carcinoma of the head and neck cell lines by human papillomavirus.* Head Neck, 2010.
48. D'Souza, G., et al., *Case-control study of human papillomavirus and oropharyngeal cancer.* N Engl J Med, 2007. **356**(19): p. 1944-56.
49. Termine, N., et al., *HPV in oral squamous cell carcinoma vs head and neck squamous cell carcinoma biopsies: a meta-analysis (1988-2007).* Ann Oncol, 2008. **19**(10): p. 1681-90.
50. Jung, A.C., et al., *Biological and clinical relevance of transcriptionally active human papillomavirus (HPV) infection in oropharynx squamous cell carcinoma.* Int J Cancer, 2010. **126**(8): p. 1882-94.
51. Munoz, N., et al., *Epidemiologic classification of human papillomavirus types associated with cervical cancer.* N Engl J Med, 2003. **348**(6): p. 518-27.
52. Marur, S., et al., *HPV-associated head and neck cancer: a virus-related cancer epidemic.* Lancet Oncol, 2010. **11**(8): p. 781-9.
53. Hammarstedt, L., et al., *Human papillomavirus as a risk factor for the increase in incidence of tonsillar cancer.* Int J Cancer, 2006. **119**(11): p. 2620-3.
54. Nasman, A., et al., *Incidence of human papillomavirus (HPV) positive tonsillar carcinoma in Stockholm, Sweden: an epidemic of viral-induced carcinoma?* Int J Cancer, 2009. **125**(2): p. 362-6.
55. Chaturvedi, A.K., et al., *Incidence trends for human papillomavirus-related and -unrelated oral squamous cell carcinomas in the United States.* J Clin Oncol, 2008. **26**(4): p. 612-9.
56. Vidal, L. and M.L. Gillison, *Human papillomavirus in HNSCC: recognition of a distinct disease type.* Hematol Oncol Clin North Am, 2008. **22**(6): p. 1125-42, vii.
57. zur Hausen, H., *Papillomaviruses causing cancer: evasion from host-cell control in early events in carcinogenesis.* J Natl Cancer Inst, 2000. **92**(9): p. 690-8.
58. Albers, A., et al., *Antitumor activity of human papillomavirus type 16 E7-specific T cells against virally infected squamous cell carcinoma of the head and neck.* Cancer Res, 2005. **65**(23): p. 11146-55.
59. McNeil, C., *HPV in oropharyngeal cancers: new data inspire hope for vaccines.* J Natl Cancer Inst, 2000. **92**(9): p. 680-1.
60. Weiss, D., M. Koopmann, and C. Rudack, *Prevalence and impact on clinicopathological characteristics of human papillomavirus-16 DNA in cervical lymph node metastases of head and neck squamous cell carcinoma.* Head Neck, 2010.
61. Weinberger, P.M., et al., *Molecular classification identifies a subset of human papillomavirus--associated oropharyngeal cancers with favorable prognosis.* J Clin Oncol, 2006. **24**(5): p. 736-47.
62. Goldenberg, D., et al., *Cystic lymph node metastasis in patients with head and neck cancer: An HPV-associated phenomenon.* Head Neck, 2008. **30**(7): p. 898-903.
63. Zhao, M., et al., *Feasibility of quantitative PCR-based saliva rinse screening of HPV for head and neck cancer.* Int J Cancer, 2005. **117**(4): p. 605-10.
64. Begum, S., et al., *Detection of human papillomavirus-16 in fine-needle aspirates to determine tumor origin in patients with metastatic squamous cell carcinoma of the head and neck.* Clin Cancer Res, 2007. **13**(4): p. 1186-91.

65. Begum, S., et al., *Detection of human papillomavirus in cervical lymph nodes: a highly effective strategy for localizing site of tumor origin.* Clin Cancer Res, 2003. **9**(17): p. 6469-75.
66. Agrawal, Y., et al., *Oral human papillomavirus infection before and after treatment for human papillomavirus 16-positive and human papillomavirus 16-negative head and neck squamous cell carcinoma.* Clin Cancer Res, 2008. **14**(21): p. 7143-50.
67. Chuang, A.Y., et al., *Presence of HPV DNA in convalescent salivary rinses is an adverse prognostic marker in head and neck squamous cell carcinoma.* Oral Oncol, 2008. **44**(10): p. 915-9.
68. Pfister, D.G. and a. al., *NCCN Clinical Practice Guidelines in Oncology - Head and Neck cancers.* www.nccn.org, 2010.
69. Wiest, T., et al., *Involvement of intact HPV16 E6/E7 gene expression in head and neck cancers with unaltered p53 status and perturbed pRb cell cycle control.* Oncogene, 2002. **21**(10): p. 1510-7.
70. Begum, S., et al., *Tissue distribution of human papillomavirus 16 DNA integration in patients with tonsillar carcinoma.* Clin Cancer Res, 2005. **11**(16): p. 5694-9.
71. Kuo, K.T., et al., *The biomarkers of human papillomavirus infection in tonsillar squamous cell carcinoma-molecular basis and predicting favorable outcome.* Mod Pathol, 2008. **21**(4): p. 376-86.
72. Smeets, S.J., et al., *A novel algorithm for reliable detection of human papillomavirus in paraffin embedded head and neck cancer specimen.* Int J Cancer, 2007. **121**(11): p. 2465-72.
73. Rotnaglova, E., et al., *HPV involvement in tonsillar cancer: Prognostic significance and clinically relevant markers.* International journal of cancer. Journal international du cancer, 2010.
74. Zhang, M.Q., S.K. El-Mofty, and R.M. Davila, *Detection of human papillomavirus-related squamous cell carcinoma cytologically and by in situ hybridization in fine-needle aspiration biopsies of cervical metastasis: a tool for identifying the site of an occult head and neck primary.* Cancer, 2008. **114**(2): p. 118-23.
75. Smith, E.M., et al., *Does pretreatment seropositivity to human papillomavirus have prognostic significance for head and neck cancers?* Cancer Epidemiol Biomarkers Prev, 2008. **17**(8): p. 2087-96.
76. Capone, R.B., et al., *Detection and quantitation of human papillomavirus (HPV) DNA in the sera of patients with HPV-associated head and neck squamous cell carcinoma.* Clin Cancer Res, 2000. **6**(11): p. 4171-5.
77. Smith, E.M., et al., *Human papillomavirus seropositivity and risks of head and neck cancer.* Int J Cancer, 2007. **120**(4): p. 825-32.
78. Braakhuis, B.J., et al., *Human papilloma virus in head and neck cancer: the need for a standardised assay to assess the full clinical importance.* Eur J Cancer, 2009. **45**(17): p. 2935-9.
79. Ang, K.K., et al., *Human papillomavirus and survival of patients with oropharyngeal cancer.* N Engl J Med, 2010. **363**(1): p. 24-35.
80. Kumar, B., et al., *EGFR, p16, HPV Titer, Bcl-xL and p53, sex, and smoking as indicators of response to therapy and survival in oropharyngeal cancer.* J Clin Oncol, 2008. **26**(19): p. 3128-37.
81. Graziano, D.F. and O.J. Finn, *Tumor antigens and tumor antigen discovery.* Cancer Treat Res, 2005. **123**: p. 89-111.
82. Srivastava, P.K. and L.J. Old, *Individually distinct transplantation antigens of chemically induced mouse tumors.* Immunol Today, 1988. **9**(3): p. 78-83.
83. Melief, C.J., et al., *Immunosurveillance of virus-induced tumors.* Cold Spring Harb Symp Quant Biol, 1989. **54 Pt 1**: p. 597-603.

84. Hislop, A.D., et al., *Cellular responses to viral infection in humans: lessons from Epstein-Barr virus.* Annu Rev Immunol, 2007. **25**: p. 587-617.
85. Kohler, G. and C. Milstein, *Continuous cultures of fused cells secreting antibody of predefined specificity.* Nature, 1975. **256**(5517): p. 495-7.
86. Hellstrom, K.E., I. Hellstrom, and J.P. Brown, *Human tumor-associated antigens identified by monoclonal antibodies.* Springer Semin Immunopathol, 1982. **5**(2): p. 127-46.
87. van der Bruggen, P., et al., *A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma.* Science, 1991. **254**(5038): p. 1643-7.
88. Renkvist, N., et al., *A listing of human tumor antigens recognized by T cells.* Cancer Immunol Immunother, 2001. **50**(1): p. 3-15.
89. Rock, K.L. and L. Shen, *Cross-presentation: underlying mechanisms and role in immune surveillance.* Immunol Rev, 2005. **207**: p. 166-83.
90. Appella, E., et al., *Synthetic antigenic peptides as a new strategy for immunotherapy of cancer.* Biomed Pept Proteins Nucleic Acids, 1995. **1**(3): p. 177-84.
91. Chesnut, R.W., et al., *Design and testing of peptide-based cytotoxic T-cell-mediated immunotherapeutics to treat infectious diseases and cancer.* Pharm Biotechnol, 1995. **6**: p. 847-74.
92. Slingluff, C.L., Jr., D.F. Hunt, and V.H. Engelhard, *Direct analysis of tumor-associated peptide antigens.* Curr Opin Immunol, 1994. **6**(5): p. 733-40.
93. Banchereau, J. and R.M. Steinman, *Dendritic cells and the control of immunity.* Nature, 1998. **392**(6673): p. 245-52.
94. Suzuki, H., et al., *T cell-dependent antibody responses against aberrantly expressed cyclin B1 protein in patients with cancer and premalignant disease.* Clin Cancer Res, 2005. **11**(4): p. 1521-6.
95. Chen, Y.M., et al., *Antibody reactivities to tumor-suppressor protein p53 and HTLV-I Tof, Rex and Tax in HTLV-I-infected people with differing clinical status.* Int J Cancer, 1997. **71**(2): p. 196-202.
96. Carlos, C.A., et al., *Human tumor antigen MUC1 is chemotactic for immature dendritic cells and elicits maturation but does not promote Th1 type immunity.* J Immunol, 2005. **175**(3): p. 1628-35.
97. Aarnoudse, C.A., et al., *Recognition of tumor glycans by antigen-presenting cells.* Curr Opin Immunol, 2006. **18**(1): p. 105-11.
98. Dunn, G.P., et al., *A critical function for type I interferons in cancer immunoediting.* Nat Immunol, 2005. **6**(7): p. 722-9.
99. Obeid, M., et al., *Calreticulin exposure dictates the immunogenicity of cancer cell death.* Nat Med, 2007. **13**(1): p. 54-61.
100. Apetoh, L., et al., *Immunogenicity of anthracyclines: moving towards more personalized medicine.* Trends Mol Med, 2008. **14**(4): p. 141-51.
101. Zitvogel, L., et al., *Immunological aspects of cancer chemotherapy.* Nat Rev Immunol, 2008. **8**(1): p. 59-73.
102. Koebel, C.M., et al., *Adaptive immunity maintains occult cancer in an equilibrium state.* Nature, 2007. **450**(7171): p. 903-7.
103. Saeland, E., et al., *The C-type lectin MGL expressed by dendritic cells detects glycan changes on MUC1 in colon carcinoma.* Cancer Immunol Immunother, 2007. **56**(8): p. 1225-36.
104. Zeng, G., et al., *Dendritic cell surface calreticulin is a receptor for NY-ESO-1: direct interactions between tumor-associated antigen and the innate immune system.* J Immunol, 2006. **177**(6): p. 3582-9.
105. Rihova, B., et al., *Cytotoxicity and immunostimulation: double attack on cancer cells with polymeric therapeutics.* Trends Biotechnol, 2009. **27**(1): p. 11-7.

106. Smyth, M.J., G.P. Dunn, and R.D. Schreiber, *Cancer immunosurveillance and immunoediting: the roles of immunity in suppressing tumor development and shaping tumor immunogenicity.* Adv Immunol, 2006. **90**: p. 1-50.
107. Russell, J.P., J.B. Engiles, and J.L. Rothstein, *Proinflammatory mediators and genetic background in oncogene mediated tumor progression.* J Immunol, 2004. **172**(7): p. 4059-67.
108. *The Nobel Prize in Physiology or Medicine 1960.* 2011; Available from: http://nobelprize.org/nobel_prizes/medicine/laureates/1960/.
109. Dunn, G.P., et al., *Cancer immunoediting: from immunosurveillance to tumor escape.* Nat Immunol, 2002. **3**(11): p. 991-8.
110. Shankaran, V., et al., *IFNgamma and lymphocytes prevent primary tumour development and shape tumour immunogenicity.* Nature, 2001. **410**(6832): p. 1107-11.
111. Dunn, G.P., L.J. Old, and R.D. Schreiber, *The three Es of cancer immunoediting.* Annu Rev Immunol, 2004. **22**: p. 329-60.
112. Roithmaier, S., et al., *Incidence of malignancies in heart and/or lung transplant recipients: a single-institution experience.* J Heart Lung Transplant, 2007. **26**(8): p. 845-9.
113. Imai, K., et al., *Natural cytotoxic activity of peripheral-blood lymphocytes and cancer incidence: an 11-year follow-up study of a general population.* Lancet, 2000. **356**(9244): p. 1795-9.
114. Galon, J., et al., *Type, density, and location of immune cells within human colorectal tumors predict clinical outcome.* Science, 2006. **313**(5795): p. 1960-4.
115. Piersma, S.J., et al., *High number of intraepithelial CD8+ tumor-infiltrating lymphocytes is associated with the absence of lymph node metastases in patients with large early-stage cervical cancer.* Cancer Res, 2007. **67**(1): p. 354-61.
116. Kohrt, H.E., et al., *Profile of immune cells in axillary lymph nodes predicts disease-free survival in breast cancer.* PLoS Med, 2005. **2**(9): p. e284.
117. Sharma, P., et al., *CD8 tumor-infiltrating lymphocytes are predictive of survival in muscle-invasive urothelial carcinoma.* Proc Natl Acad Sci U S A, 2007. **104**(10): p. 3967-72.
118. Dave, S.S., et al., *Prediction of survival in follicular lymphoma based on molecular features of tumor-infiltrating immune cells.* N Engl J Med, 2004. **351**(21): p. 2159-69.
119. Wahlin, B.E., et al., *CD8+ T-cell content in diagnostic lymph nodes measured by flow cytometry is a predictor of survival in follicular lymphoma.* Clin Cancer Res, 2007. **13**(2 Pt 1): p. 388-97.
120. Rabinovich, G.A., D. Gabrilovich, and E.M. Sotomayor, *Immunosuppressive strategies that are mediated by tumor cells.* Annu Rev Immunol, 2007. **25**: p. 267-96.
121. Teicher, B.A., *Transforming growth factor-beta and the immune response to malignant disease.* Clin Cancer Res, 2007. **13**(21): p. 6247-51.
122. Houston, A., et al., *Fas ligand mediates immune privilege and not inflammation in human colon cancer, irrespective of TGF-beta expression.* Br J Cancer, 2003. **89**(7): p. 1345-51.
123. Uyttenhove, C., et al., *Evidence for a tumoral immune resistance mechanism based on tryptophan degradation by indoleamine 2,3-dioxygenase.* Nat Med, 2003. **9**(10): p. 1269-74.
124. Muller, A.J. and G.C. Prendergast, *Indoleamine 2,3-dioxygenase in immune suppression and cancer.* Curr Cancer Drug Targets, 2007. **7**(1): p. 31-40.
125. Munn, D.H., et al., *Prevention of allogeneic fetal rejection by tryptophan catabolism.* Science, 1998. **281**(5380): p. 1191-3.
126. Munn, D.H., et al., *Potential regulatory function of human dendritic cells expressing indoleamine 2,3-dioxygenase.* Science, 2002. **297**(5588): p. 1867-70.
127. Zou, W., *Regulatory T cells, tumour immunity and immunotherapy.* Nat Rev Immunol, 2006. **6**(4): p. 295-307.

128. Bergmann, C., et al., *Expansion and characteristics of human T regulatory type 1 cells in co-cultures simulating tumor microenvironment.* Cancer Immunol Immunother, 2007. **56**(9): p. 1429-42.
129. Chikamatsu, K., et al., *Relationships between regulatory T cells and CD8+ effector populations in patients with squamous cell carcinoma of the head and neck.* Head Neck, 2007. **29**(2): p. 120-7.
130. Fecci, P.E., et al., *Increased regulatory T-cell fraction amidst a diminished CD4 compartment explains cellular immune defects in patients with malignant glioma.* Cancer Res, 2006. **66**(6): p. 3294-302.
131. Sica, A., P. Allavena, and A. Mantovani, *Cancer related inflammation: the macrophage connection.* Cancer Lett, 2008. **267**(2): p. 204-15.
132. Mantovani, A., et al., *Cancer-related inflammation.* Nature, 2008. **454**(7203): p. 436-44.
133. Finn, O.J., *Cancer immunology.* N Engl J Med, 2008. **358**(25): p. 2704-15.
134. Sakaguchi, S. and N. Sakaguchi, *Regulatory T cells in immunologic self-tolerance and autoimmune disease.* Int Rev Immunol, 2005. **24**(3-4): p. 211-26.
135. Kapp, J.A., *Special regulatory T-cell review: Suppressors regulated but unsuppressed.* Immunology, 2008. **123**(1): p. 28-32.
136. Pomie, C., I. Menager-Marcq, and J.P. van Meerwijk, *Murine CD8(+) regulatory T lymphocytes: The new era.* Hum Immunol, 2008. **69**(11): p. 708-14.
137. Sakaguchi, S., *Naturally arising Foxp3-expressing CD25+CD4+ regulatory T cells in immunological tolerance to self and non-self.* Nat Immunol, 2005. **6**(4): p. 345-52.
138. Campbell, D.J. and S.F. Ziegler, *FOXP3 modifies the phenotypic and functional properties of regulatory T cells.* Nat Rev Immunol, 2007. **7**(4): p. 305-10.
139. Vignali, D.A., L.W. Collison, and C.J. Workman, *How regulatory T cells work.* Nat Rev Immunol, 2008. **8**(7): p. 523-32.
140. Sakaguchi, S., et al., *Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases.* J Immunol, 1995. **155**(3): p. 1151-64.
141. Wolf, A.M., et al., *Increase of regulatory T cells in the peripheral blood of cancer patients.* Clin Cancer Res, 2003. **9**(2): p. 606-12.
142. Woo, E.Y., et al., *Regulatory CD4(+)CD25(+) T cells in tumors from patients with early-stage non-small cell lung cancer and late-stage ovarian cancer.* Cancer Res, 2001. **61**(12): p. 4766-72.
143. Strauss, L., et al., *The frequency and suppressor function of CD4+CD25highFoxp3+ T cells in the circulation of patients with squamous cell carcinoma of the head and neck.* Clin Cancer Res, 2007. **13**(21): p. 6301-11.
144. Boucek, J., et al., *Regulatory T cells and their prognostic value for patients with Squamous Cell Carcinoma of the Head and Neck.* Journal of Cellular and Molecular Medicine, In press.
145. Mandapathil, M., et al., *Increased ectonucleotidase expression and activity in regulatory T cells of patients with head and neck cancer.* Clin Cancer Res, 2009. **15**(20): p. 6348-57.
146. Groux, H. and F. Cottrez, *The complex role of interleukin-10 in autoimmunity.* J Autoimmun, 2003. **20**(4): p. 281-5.
147. Vignali, D., *How many mechanisms do regulatory T cells need?* Eur J Immunol, 2008. **38**(4): p. 908-11.
148. Strauss, L., C. Bergmann, and T.L. Whiteside, *Functional and phenotypic characteristics of CD4+CD25highFoxp3+ Treg clones obtained from peripheral blood of patients with cancer.* Int J Cancer, 2007. **121**(11): p. 2473-83.
149. Martin, F., et al., *Human FOXP3 and cancer.* Oncogene, 2010. **29**(29): p. 4121-9.
150. Randolph, D.A. and C.G. Fathman, *Cd4+Cd25+ regulatory T cells and their therapeutic potential.* Annu Rev Med, 2006. **57**: p. 381-402.

151. Tai, X., et al., *CD28 costimulation of developing thymocytes induces Foxp3 expression and regulatory T cell differentiation independently of interleukin 2.* Nat Immunol, 2005. **6**(2): p. 152-62.
152. Kronenberg, M. and A. Rudensky, *Regulation of immunity by self-reactive T cells.* Nature, 2005. **435**(7042): p. 598-604.
153. Liu, W., et al., *CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells.* J Exp Med, 2006. **203**(7): p. 1701-11.
154. Strauss, L., et al., *A unique subset of CD4+CD25highFoxp3+ T cells secreting interleukin-10 and transforming growth factor-beta1 mediates suppression in the tumor microenvironment.* Clin Cancer Res, 2007. **13**(15 Pt 1): p. 4345-54.
155. Hilchey, S.P., et al., *Follicular lymphoma intratumoral CD4+CD25+GITR+ regulatory T cells potently suppress CD3/CD28-costimulated autologous and allogeneic CD8+CD25- and CD4+CD25- T cells.* J Immunol, 2007. **178**(7): p. 4051-61.
156. Takahashi, T., et al., *Immunologic self-tolerance maintained by CD25+CD4+ naturally anergic and suppressive T cells: induction of autoimmune disease by breaking their anergic/suppressive state.* Int Immunol, 1998. **10**(12): p. 1969-80.
157. Thornton, A.M. and E.M. Shevach, *CD4+CD25+ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production.* J Exp Med, 1998. **188**(2): p. 287-96.
158. Jonuleit, H., et al., *Identification and functional characterization of human CD4(+)CD25(+) T cells with regulatory properties isolated from peripheral blood.* J Exp Med, 2001. **193**(11): p. 1285-94.
159. Grossman, W.J., et al., *Differential expression of granzymes A and B in human cytotoxic lymphocyte subsets and T regulatory cells.* Blood, 2004. **104**(9): p. 2840-8.
160. Zhao, D.M., et al., *Activated CD4+CD25+ T cells selectively kill B lymphocytes.* Blood, 2006. **107**(10): p. 3925-32.
161. Cao, X., et al., *Granzyme B and perforin are important for regulatory T cell-mediated suppression of tumor clearance.* Immunity, 2007. **27**(4): p. 635-46.
162. Shevach, E.M., *Mechanisms of foxp3+ T regulatory cell-mediated suppression.* Immunity, 2009. **30**(5): p. 636-45.
163. Bopp, T., et al., *Cyclic adenosine monophosphate is a key component of regulatory T cell-mediated suppression.* J Exp Med, 2007. **204**(6): p. 1303-10.
164. Deaglio, S., et al., *Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression.* J Exp Med, 2007. **204**(6): p. 1257-65.
165. Irla, M., et al., *MHC class II-restricted antigen presentation by plasmacytoid dendritic cells inhibits T cell-mediated autoimmunity.* J Exp Med, 2010. **207**(9): p. 1891-905.
166. Fallarino, F., et al., *Modulation of tryptophan catabolism by regulatory T cells.* Nat Immunol, 2003. **4**(12): p. 1206-12.
167. Mellor, A.L. and D.H. Munn, *IDO expression by dendritic cells: tolerance and tryptophan catabolism.* Nat Rev Immunol, 2004. **4**(10): p. 762-74.
168. Liu, Y., et al., *A critical function for TGF-beta signaling in the development of natural CD4+CD25+Foxp3+ regulatory T cells.* Nat Immunol, 2008. **9**(6): p. 632-40.
169. Verhasselt, V., et al., *Breast milk-mediated transfer of an antigen induces tolerance and protection from allergic asthma.* Nat Med, 2008. **14**(2): p. 170-5.
170. Whiteside, T.L., *Anti-tumor vaccines in head and neck cancer: targeting immune responses to the tumor.* Curr Cancer Drug Targets, 2007. **7**(7): p. 633-42.
171. Erdman, S.E., et al., *CD4+CD25+ regulatory lymphocytes induce regression of intestinal tumors in ApcMin/+ mice.* Cancer Res, 2005. **65**(10): p. 3998-4004.
172. Erdman, S.E., et al., *CD4+ CD25+ regulatory T lymphocytes inhibit microbially induced colon cancer in Rag2-deficient mice.* Am J Pathol, 2003. **162**(2): p. 691-702.
173. Salama, P., et al., *Tumor-infiltrating FOXP3+ T regulatory cells show strong prognostic significance in colorectal cancer.* J Clin Oncol, 2009. **27**(2): p. 186-92.

174. Kobayashi, N., et al., *FOXP3+ regulatory T cells affect the development and progression of hepatocarcinogenesis.* Clin Cancer Res, 2007. **13**(3): p. 902-11.
175. Hiraoka, N., et al., *Prevalence of FOXP3+ regulatory T cells increases during the progression of pancreatic ductal adenocarcinoma and its premalignant lesions.* Clin Cancer Res, 2006. **12**(18): p. 5423-34.
176. Carreras, J., et al., *High numbers of tumor-infiltrating FOXP3-positive regulatory T cells are associated with improved overall survival in follicular lymphoma.* Blood, 2006. **108**(9): p. 2957-64.
177. Lee, A.M., et al., *Number of CD4+ cells and location of forkhead box protein P3-positive cells in diagnostic follicular lymphoma tissue microarrays correlates with outcome.* J Clin Oncol, 2006. **24**(31): p. 5052-9.
178. Alvaro, T., et al., *Outcome in Hodgkin's lymphoma can be predicted from the presence of accompanying cytotoxic and regulatory T cells.* Clin Cancer Res, 2005. **11**(4): p. 1467-73.
179. Badoual, C., et al., *Prognostic value of tumor-infiltrating CD4+ T-cell subpopulations in head and neck cancers.* Clin Cancer Res, 2006. **12**(2): p. 465-72.
180. Petersen, R.P., et al., *Tumor infiltrating Foxp3+ regulatory T-cells are associated with recurrence in pathologic stage I NSCLC patients.* Cancer, 2006. **107**(12): p. 2866-72.
181. Leffers, N., et al., *Prognostic significance of tumor-infiltrating T-lymphocytes in primary and metastatic lesions of advanced stage ovarian cancer.* Cancer Immunol Immunother, 2009. **58**(3): p. 449-59.
182. Elpek, K.G., et al., *CD4+CD25+ T regulatory cells dominate multiple immune evasion mechanisms in early but not late phases of tumor development in a B cell lymphoma model.* J Immunol, 2007. **178**(11): p. 6840-8.
183. Kovar, M., et al., *Overcoming immunoescape mechanisms of BCL1 leukemia and induction of CD8+ T-cell-mediated BCL1-specific resistance in mice cured by targeted polymer-bound doxorubicin.* Cancer Res, 2008. **68**(23): p. 9875-83.
184. Gao, Q., et al., *Intratumoral balance of regulatory and cytotoxic T cells is associated with prognosis of hepatocellular carcinoma after resection.* J Clin Oncol, 2007. **25**(18): p. 2586-93.
185. Sato, E., et al., *Intraepithelial CD8+ tumor-infiltrating lymphocytes and a high CD8+/regulatory T cell ratio are associated with favorable prognosis in ovarian cancer.* Proc Natl Acad Sci U S A, 2005. **102**(51): p. 18538-43.
186. Walker, M.R., et al., *Induction of FoxP3 and acquisition of T regulatory activity by stimulated human CD4+CD25- T cells.* J Clin Invest, 2003. **112**(9): p. 1437-43.
187. Roncador, G., et al., *Analysis of FOXP3 protein expression in human CD4+CD25+ regulatory T cells at the single-cell level.* Eur J Immunol, 2005. **35**(6): p. 1681-91.
188. Cosmi, L., et al., *Human CD8+CD25+ thymocytes share phenotypic and functional features with CD4+CD25+ regulatory thymocytes.* Blood, 2003. **102**(12): p. 4107-14.
189. Badoual, C., et al., *The soluble alpha chain of interleukin-15 receptor: a proinflammatory molecule associated with tumor progression in head and neck cancer.* Cancer Res, 2008. **68**(10): p. 3907-14.
190. Schottelius, A.J. and H. Dinter, *Cytokines, NF-kappaB, microenvironment, intestinal inflammation and cancer.* Cancer Treat Res, 2006. **130**: p. 67-87.
191. Maloy, K.J., et al., *CD4+CD25+ T(R) cells suppress innate immune pathology through cytokine-dependent mechanisms.* J Exp Med, 2003. **197**(1): p. 111-9.
192. Venet, F., et al., *Human CD4+CD25+ regulatory T lymphocytes inhibit lipopolysaccharide-induced monocyte survival through a Fas/Fas ligand-dependent mechanism.* J Immunol, 2006. **177**(9): p. 6540-7.
193. Grossman, W.J., et al., *Human T regulatory cells can use the perforin pathway to cause autologous target cell death.* Immunity, 2004. **21**(4): p. 589-601.
194. Plzak, J., et al., *Galectin-3 - an emerging prognostic indicator in advanced head and neck carcinoma.* Eur J Cancer, 2004. **40**(15): p. 2324-30.

195. Cada, Z., et al., *Nucleostemin expression in squamous cell carcinoma of the head and neck.* Anticancer Res, 2007. **27**(5A): p. 3279-84.
196. Klozar, J., et al., *HPV status and regional metastasis in the prognosis of oral and oropharyngeal cancer.* Eur Arch Otorhinolaryngol, 2008. **265 Suppl 1**: p. S75-82.
197. Smetana, K., Jr., et al., *Human hair follicle and interfollicular keratinocyte reactivity to mouse HPV16-transformed cells: an in vitro study.* Oncol Rep, 2008. **20**(1): p. 75-80.
198. Bergmann, C., et al., *T regulatory type 1 cells in squamous cell carcinoma of the head and neck: mechanisms of suppression and expansion in advanced disease.* Clin Cancer Res, 2008. **14**(12): p. 3706-15.
199. Xu, L., et al., *Depletion of CD4(+)CD25(high) regulatory T cells from tumor infiltrating lymphocytes predominantly induces Th1 type immune response in vivo which inhibits tumor growth in adoptive immunotherapy.* Cancer Biol Ther, 2009. **8**(1).
200. Curtin, J.F., et al., *Treg depletion inhibits efficacy of cancer immunotherapy: implications for clinical trials.* PLoS ONE, 2008. **3**(4): p. e1983.
201. Prince, M.E., et al., *Identification of a subpopulation of cells with cancer stem cell properties in head and neck squamous cell carcinoma.* Proc Natl Acad Sci U S A, 2007. **104**(3): p. 973-8.
202. Burkert, J., N.A. Wright, and M.R. Alison, *Stem cells and cancer: an intimate relationship.* J Pathol, 2006. **209**(3): p. 287-97.
203. Monroe, M.M., et al., *Cancer stem cells in head and neck squamous cell carcinoma.* J Oncol, 2011. **2011**: p. 762780.
204. Dalerba, P., R.W. Cho, and M.F. Clarke, *Cancer stem cells: models and concepts.* Annu Rev Med, 2007. **58**: p. 267-84.
205. Al-Hajj, M., et al., *Therapeutic implications of cancer stem cells.* Curr Opin Genet Dev, 2004. **14**(1): p. 43-7.
206. Visvader, J.E. and G.J. Lindeman, *Cancer stem cells in solid tumours: accumulating evidence and unresolved questions.* Nat Rev Cancer, 2008. **8**(10): p. 755-68.
207. Dean, M., *Cancer stem cells: Implications for cancer causation and therapy resistance.* Discov Med, 2005. **5**(27): p. 278-82.
208. Dingli, D. and F. Michor, *Successful therapy must eradicate cancer stem cells.* Stem Cells, 2006. **24**(12): p. 2603-10.
209. Dean, M., T. Fojo, and S. Bates, *Tumour stem cells and drug resistance.* Nat Rev Cancer, 2005. **5**(4): p. 275-84.
210. Wu, C. and B.A. Alman, *Side population cells in human cancers.* Cancer Lett, 2008. **268**(1): p. 1-9.
211. Challen, G.A. and M.H. Little, *A side order of stem cells: the SP phenotype.* Stem Cells, 2006. **24**(1): p. 3-12.
212. Haraguchi, N., et al., *Characterization of a side population of cancer cells from human gastrointestinal system.* Stem Cells, 2006. **24**(3): p. 506-13.
213. Zhou, S., et al., *The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype.* Nat Med, 2001. **7**(9): p. 1028-34.
214. Hirschmann-Jax, C., et al., *A distinct "side population" of cells with high drug efflux capacity in human tumor cells.* Proc Natl Acad Sci U S A, 2004. **101**(39): p. 14228-33.
215. Chiba, T., et al., *Side population purified from hepatocellular carcinoma cells harbors cancer stem cell-like properties.* Hepatology, 2006. **44**(1): p. 240-51.
216. Szotek, P.P., et al., *Ovarian cancer side population defines cells with stem cell-like characteristics and Mullerian Inhibiting Substance responsiveness.* Proc Natl Acad Sci U S A, 2006. **103**(30): p. 11154-9.
217. Kondo, T., T. Setoguchi, and T. Taga, *Persistence of a small subpopulation of cancer stem-like cells in the C6 glioma cell line.* Proc Natl Acad Sci U S A, 2004. **101**(3): p. 781-6.

218. Zhang, X., et al., *Understanding metastatic SCCHN cells from unique genotypes to phenotypes with the aid of an animal model and DNA microarray analysis.* Clin Exp Metastasis, 2006. **23**(3-4): p. 209-22.
219. Zhang, X., et al., *A lymph node metastatic mouse model reveals alterations of metastasis-related gene expression in metastatic human oral carcinoma sublines selected from a poorly metastatic parental cell line.* Cancer, 2002. **95**(8): p. 1663-72.
220. Reya, T. and H. Clevers, *Wnt signalling in stem cells and cancer.* Nature, 2005. **434**(7035): p. 843-50.
221. Song, J., et al., *Characterization of side populations in HNSCC: highly invasive, chemoresistant and abnormal Wnt signaling.* PLoS One, 2010. **5**(7): p. e11456.
222. Specenier, P. and J.B. Vermorken, *Advances in the systemic treatment of head and neck cancers.* Curr Opin Oncol, 2010. **22**(3): p. 200-5.
223. Specenier, P.M. and J.B. Vermorken, *Current concepts for the management of head and neck cancer: chemotherapy.* Oral Oncol, 2009. **45**(4-5): p. 409-15.
224. Vermorken, J.B., et al., *Platinum-based chemotherapy plus cetuximab in head and neck cancer.* N Engl J Med, 2008. **359**(11): p. 1116-27.
225. Airoldi, M., et al., *Paclitaxel and pegylated liposomal doxorubicin in recurrent head and neck cancer: clinical and unexpected pharmacokinetic interactions.* Anticancer Res, 2008. **28**(4C): p. 2519-27.
226. Jehn, C.F., et al., *First safety and response results of a randomized phase III study with liposomal platin in the treatment of advanced squamous cell carcinoma of the head and neck (SCCHN).* Anticancer Res, 2008. **28**(6B): p. 3961-4.
227. Cohen, E.E., et al., *Factors associated with clinical benefit from epidermal growth factor receptor inhibitors in recurrent and metastatic squamous cell carcinoma of the head and neck.* Oral Oncol, 2009. **45**(10): p. e155-60.
228. Nutting, C.M., et al., *Phase II study of 3-AP Triapine in patients with recurrent or metastatic head and neck squamous cell carcinoma.* Ann Oncol, 2009. **20**(7): p. 1275-9.
229. Hanrahan, E.O., et al., *A phase II study of Lonafarnib (SCH66336) in patients with chemorefractory, advanced squamous cell carcinoma of the head and neck.* Am J Clin Oncol, 2009. **32**(3): p. 274-9.
230. Blumenschein, G.R., Jr., et al., *Phase II trial of the histone deacetylase inhibitor vorinostat (Zolinza, suberoylanilide hydroxamic acid, SAHA) in patients with recurrent and/or metastatic head and neck cancer.* Invest New Drugs, 2008. **26**(1): p. 81-7.
231. Recchia, F., et al., *Maintenance immunotherapy in recurrent or metastatic squamous cell carcinoma of the head and neck.* J Immunother, 2008. **31**(4): p. 413-9.
232. Sauter, A., et al., *Pharmacokinetics, immunogenicity and safety of bivatuzumab mertansine, a novel CD44v6-targeting immunoconjugate, in patients with squamous cell carcinoma of the head and neck.* Int J Oncol, 2007. **30**(4): p. 927-35.
233. Riechelmann, H., et al., *Phase I trial with the CD44v6-targeting immunoconjugate bivatuzumab mertansine in head and neck squamous cell carcinoma.* Oral Oncol, 2008. **44**(9): p. 823-9.
234. Bernier, J., *Incorporation of molecularly targeted agents in the primary treatment of squamous cell carcinomas of the head and neck.* Hematol Oncol Clin North Am, 2008. **22**(6): p. 1193-208, ix.
235. Brieger, J., et al., *Evaluation of microsatellite amplifications at chromosomal locus 3q26 as surrogate marker for premalignant changes in mucosa surrounding head and neck squamous cell carcinoma.* Cancer Genet Cytogenet, 2006. **167**(1): p. 26-31.
236. Boucek, J., et al., *Regulatory T cells and their prognostic value for patients with Squamous Cell Carcinoma of the Head and Neck.* J Cell Mol Med, 2009.
237. Ehrlich, P., *Studies in immunity.* Plenum, New York, 1906.
238. Kantarjian, H., et al., *Hematologic and cytogenetic responses to imatinib mesylate in chronic myelogenous leukemia.* N Engl J Med, 2002. **346**(9): p. 645-52.

239. Druker, B.J., et al., *Effects of a selective inhibitor of the Abl tyrosine kinase on the growth of Bcr-Abl positive cells.* Nat Med, 1996. **2**(5): p. 561-6.
240. Cohen, S., *Isolation of a mouse submaxillary gland protein accelerating incisor eruption and eyelid opening in the new-born animal.* J Biol Chem, 1962. **237**: p. 1555-62.
241. Cohen, S., G. Carpenter, and L. King, Jr., *Epidermal growth factor-receptor-protein kinase interactions. Co-purification of receptor and epidermal growth factor-enhanced phosphorylation activity.* J Biol Chem, 1980. **255**(10): p. 4834-42.
242. DeVita, V.T., Jr. and E. Chu, *A history of cancer chemotherapy.* Cancer Res, 2008. **68**(21): p. 8643-53.
243. Chabner, B.A. and T.G. Roberts, Jr., *Timeline: Chemotherapy and the war on cancer.* Nat Rev Cancer, 2005. **5**(1): p. 65-72.
244. Baselga, J., et al., *Phase II multicenter study of the antiepidermal growth factor receptor monoclonal antibody cetuximab in combination with platinum-based chemotherapy in patients with platinum-refractory metastatic and/or recurrent squamous cell carcinoma of the head and neck.* J Clin Oncol, 2005. **23**(24): p. 5568-77.
245. Bonner, J.A., et al., *Radiotherapy plus cetuximab for squamous-cell carcinoma of the head and neck.* N Engl J Med, 2006. **354**(6): p. 567-78.
246. Burtness, B., et al., *Phase III Randomized Trial of Cisplatin Plus Placebo Compared With Cisplatin Plus Cetuximab in Metastatic/Recurrent Head and Neck Cancer: An Eastern Cooperative Oncology Group Study.* J Clin Oncol, 2005. **23**(34): p. 8646-8654.
247. Vermorken, J.B., et al., *Open-Label, Uncontrolled, Multicenter Phase II Study to Evaluate the Efficacy and Toxicity of Cetuximab As a Single Agent in Patients With Recurrent and/or Metastatic Squamous Cell Carcinoma of the Head and Neck Who Failed to Respond to Platinum-Based Therapy.* J Clin Oncol, 2007. **25**(16): p. 2171-2177.
248. Herbst, R.S., M. Fukuoka, and J. Baselga, *Gefitinib--a novel targeted approach to treating cancer.* Nat Rev Cancer, 2004. **4**(12): p. 956-65.
249. Bonner, J.A., et al., *Radiotherapy plus cetuximab for locoregionally advanced head and neck cancer: 5-year survival data from a phase 3 randomised trial, and relation between cetuximab-induced rash and survival.* Lancet Oncol, 2010. **11**(1): p. 21-8.
250. Bernier, J., S.M. Bentzen, and J.B. Vermorken, *Molecular therapy in head and neck oncology.* Nat Rev Clin Oncol, 2009. **6**(5): p. 266-77.
251. Kopecek, J. and H. Bazilova, *Poly[N-(2-hydroxypropyl)methacrylamide]-I. Radical polymerization and copolymerization.* European Polymer Journal, 1973. **9**(1): p. 7-14.
252. Rihova, B. and I. Riha, *Immunological problems of polymer-bound drugs.* Crit Rev Ther Drug Carrier Syst, 1985. **1**(4): p. 311-74.
253. Rihova, B., et al., *Antibody-directed affinity therapy applied to the immune system: in vivo effectiveness and limited toxicity of daunomycin conjugated to HPMA copolymers and targeting antibody.* Clin Immunol Immunopathol, 1988. **46**(1): p. 100-14.
254. Rihova, B., *Immunomodulating activities of soluble synthetic polymer-bound drugs.* Adv Drug Deliv Rev, 2002. **54**(5): p. 653-74.
255. Hortobagyi, G.N., *Anthracyclines in the treatment of cancer. An overview.* Drugs, 1997. **54 Suppl 4**: p. 1-7.
256. Rossmann, P., et al., *Morphology of rat kidney and thymus after native and antibody-coupled cyclosporin A application (reduced toxicity of targeted drug).* Folia Microbiol (Praha), 1997. **42**(3): p. 277-87.
257. St'astny, M., et al., *Abnormal differentiation of thymocytes induced by free cyclosporine is avoided when cyclosporine bound to N-(2-hydroxypropyl)methacrylamide copolymer carrier is used.* Transplantation, 1997. **63**(12): p. 1818-27.
258. Chytil, P., et al., *New HPMA copolymer-based drug carriers with covalently bound hydrophobic substituents for solid tumour targeting.* J Control Release, 2008. **127**(2): p. 121-30.

259. Rihova, B., et al., *Doxorubicin release is not a prerequisite for the in vitro cytotoxicity of HPMA-based pharmaceuticals: in vitro effect of extra drug-free GlyPheLeuGly sequences.* J Control Release, 2008. **127**(2): p. 110-20.
260. Rejmanova, P., et al., *Stability in rat plasma and serum of lysosomally degradable oligopeptide sequences in N-(2-hydroxypropyl) methacrylamide copolymers.* Biomaterials, 1985. **6**(1): p. 45-8.
261. Etrych, T., et al., *New HPMA copolymers containing doxorubicin bound via pH-sensitive linkage: synthesis and preliminary in vitro and in vivo biological properties.* J Control Release, 2001. **73**(1): p. 89-102.
262. Rihova, B., et al., *Doxorubicin bound to a HPMA copolymer carrier through hydrazone bond is effective also in a cancer cell line with a limited content of lysosomes.* J Control Release, 2001. **74**(1-3): p. 225-32.
263. Mrkvan, T., et al., *Chemotherapy based on HPMA copolymer conjugates with pH-controlled release of doxorubicin triggers anti-tumor immunity.* J Control Release, 2005. **110**(1): p. 119-29.
264. Maeda, H., *The enhanced permeability and retention (EPR) effect in tumor vasculature: the key role of tumor-selective macromolecular drug targeting.* Adv Enzyme Regul, 2001. **41**: p. 189-207.
265. Matsumura, Y. and H. Maeda, *A new concept for macromolecular therapeutics in cancer chemotherapy: mechanism of tumoritropic accumulation of proteins and the antitumor agent smancs.* Cancer Res, 1986. **46**(12 Pt 1): p. 6387-92.
266. Kopecek, J., et al., *HPMA copolymer-anticancer drug conjugates: design, activity, and mechanism of action.* Eur J Pharm Biopharm, 2000. **50**(1): p. 61-81.
267. Kovar, M., et al., *HPMA copolymer-bound doxorubicin targeted to tumor-specific antigen of BCL1 mouse B cell leukemia.* J Control Release, 2003. **92**(3): p. 315-30.
268. Nan, A., et al., *Water-soluble polymers for targeted drug delivery to human squamous carcinoma of head and neck.* Journal of Drug Targeting, 2005. **13**(3): p. 189-197.
269. Omelyanenko, V., et al., *HPMA copolymer-anticancer drug-OV-TL16 antibody conjugates. II. Processing in epithelial ovarian carcinoma cells in vitro.* Int J Cancer, 1998. **75**(4): p. 600-8.
270. Liu, J., et al., *Biorecognition and subcellular trafficking of HPMA copolymer-anti-PSMA antibody conjugates by prostate cancer cells.* Mol Pharm, 2009. **6**(3): p. 959-70.
271. Sirova, M., et al., *Treatment with HPMA copolymer-based doxorubicin conjugate containing human immunoglobulin induces long-lasting systemic anti-tumour immunity in mice.* Cancer Immunol Immunother, 2007. **56**(1): p. 35-47.
272. Kovar, M., et al., *Overcoming Immunoescape Mechanisms of BCL1 Leukemia and Induction of CD8+ T-Cell-Mediated BCL1-Specific Resistance in Mice Cured by Targeted Polymer-Bound Doxorubicin.* Cancer Res, 2008. **68**(23): p. 9875-9883.
273. Rihova, B. and M. Kovar, *Immunogenicity and immunomodulatory properties of HPMA-based polymers.* Adv Drug Deliv Rev, 2010. **62**(2): p. 184-91.

Přehled vlastní publikační aktivity:

Časopisy s IF:

1. Regulatory T cells and their prognostic value for patients with squamous cell carcinoma of the head and neck.

   Boucek J, Mrkvan T, Chovanec M, Kuchar M, Betka J, Boucek V, Hladikova M, Betka J, Eckschlager T, Rihova B.

   J Cell Mol Med. 2010 Jan;14(1-2):426-33. Epub 2009 Jan 14.

   IF = 5,228

2. Doxorubicin release is not a prerequisite for the in vitro cytotoxicity of HPMA-based pharmaceuticals: in vitro effect of extra drug-free GlyPheLeuGly sequences.

   Ríhová B, Strohalm J, Hovorka O, Subr V, Etrych T, Chytil P, Pola R, Plocová D, Boucek J, Ulbrich K.

   J Control Release. 2008 Apr 21;127(2):110-20. Epub 2008 Jan 16.

   IF = 5,949

3. New HPMA copolymer-based drug carriers with covalently bound hydrophobic substituents for solid tumour targeting.

   Chytil P, Etrych T, Konák C, Sírová M, Mrkvan T, Boucek J, Ríhová B, Ulbrich K.

   J Control Release. 2008 Apr 21;127(2):121-30. Epub 2008 Jan 30.

   IF = 5,949

4. Nucleostemin expression in squamous cell carcinoma of the head and neck.

   Cada Z, Boucek J, Dvoranková B, Chovanec M, Plzák J, Kodet R, Betka J, Pinot GL, Gabius HJ, Smetana K Jr.

   Anticancer Res. 2007 Sep-Oct;27(5A):3279-84.

   IF = 1,428

5. Marker profiling of normal keratinocytes identifies the stroma from squamous cell carcinoma of the oral cavity as a modulatory microenvironment in co-culture.

   Lacina L, Dvoránkova B, Smetana K Jr, Chovanec M, Plzák J, Tachezy R, Kideryová L, Kucerová L, Cada Z, Boucek J, Kodet R, André S, Gabius HJ.

   Int J Radiat Biol. 2007 Nov-Dec;83(11-12):837-48.

   IF = 1,842

6. Nuclear presence of adhesion-/growth-regulatory galectins in normal/malignant cells of squamous epithelial origin.

   Smetana K Jr, Dvoránková B, Chovanec M, Boucek J, Klíma J, Motlík J, Lensch M, Kaltner H, André S, Gabius HJ.

   Histochem Cell Biol. 2006 Jan;125(1-2):171-82. Epub 2005 Oct 28.

   IF = 3,021

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Časopisy bez IF:

1) Jaroslav Kraus, Martin Chovanec, Jan Bouček – ***Karcinom hrtanu – současné trendy*** – Lékařské listy 2.9.2006

2) Martin Mašek, Jan Bouček, Karel Cvachovec, Jan Betka (25.9.2006) – ***Epiglotitis – Zkušenosti s pacienty hospitalizovanými na RES FN Motol*** – Praktický lékař, 2006, 86, č.9, s. 519-521

3) Jan Kastner, Jan Bouček, Jaroslav Betka, Jan Plzák, Marie Jáchymová, Jan Betka - ***Časná kancerogeneze u spinocelulárního karcinomu hlavy a krku (Early cancerogenesis in head and neck squamous cell carcinoma)*** **-** Prakt. Lék. 2008, 88, No. 9, pp. 501-505

4) Jan Bouček, Jaroslav Betka, Jan Kastner, Jan Betka, Tomáš Eckschlager, Blanka Říhová - ***Regulační T lymfocyty a jejich význam pro zhoubné novotvary hlavy a krku (Regulatory T cells and their prognostic value for head and neck oncology)*** **–** Prakt. Lék. 2009, 89, No. 1, pp. 16-19

5) Kastner J., Bouček J., Taudy M., Lisý J., Betka J. - **Komplikovaná akutní rinosinusitida (Complicated acute rhinosinusitis) -** Prakt. Lék. 2009, 89, No. 12, pp. 689-691

6) J. Boucek, J. Kastner, J. Skrivan, E. Grosso, B. Gibelli, G. Giugliano, J. Betka - **Occult thyroid carcinoma -** Acta Otorhinolaryngologica Italica, 2009; 29:296-304

Prezentace na kongresech, sympoziích:

a) 3rd International Conference on Tumor Microenvironment: Progression, Therapy & Prevention, Prague, Czech Republic, October 12–16, 2004 – *O. Hovorka, J.Strohlam, J. Boucek, T. Etrych, V. Subr, K. Ulbrich, B. Ríhová - Mechanism of Action Of HPMA-based Non-Targeted Polymeric Prodrugs* – poster

b) Basic Aspects of Tumor Immunology II, Keystone, Colorado, USA, March 19 – 24, 2005 – *Jan Boucek, Blanka Rihova, Jan Betka, Jiri Strohalm, Dana Plocova, Vladimir Subr, Karel Ulbrich – Targeted polymeric anticancer drugs based on HPMA effectively inhibit the growth of FaDu tumor cancer cell line (Squamous cell carcinoma)* – poster

c) 6. studentská vědecká konference 1.LFUK, Praha, Česká republika, 23. května 2005 - *Jan Bouček, Jan Betka, Karel Ulbrich, Blanka Říhová – Směrované polymerní konjugáty v terapii spinocelulárních karcinomů hlavy a krku* – přednáška

d) 68. kongres České společnosti pro otorinolaryngologii a chirurgii hlavy a krku, Brno, Česká republika, 16. – 18. června 2005 – *Jan Bouček, Jan Betka, Jiří Strohalm, Dana Plocová, Vladimír Šubr, Karel Ulbrich, Blanka Říhová – Terapie spinocelulárních karcinomů hlavy a krku polymerními konjugáty směrovanými proti EGFR* – přednáška

e) XVIII World Congress of International Federation Oto-Rhino-Laryngological Societies, Roma, Italy, 25.6. – 30.6.2005 - *Jan Boucek, Jan Betka, Jiri Strohalm, Dana Plocova, Vladimir Subr, Karel Ulbrich, Blanka Rihova - Treating Head And Neck Cancer With Polymeric Conjugates Targeted to EGFR* – poster

f) Third International Nanomedicine and Drug Delivery Symposium – University of Maryland, Baltimore, Maryland, USA September 26-27, 2005 - *J. Boucek, J. Betka, J. Strohalm, D.Plocova, V. Subr, K. Ulbrich, B. Rihova - Treating Head And Neck Cancer With Targeted Polymeric Conjugates* – poster

g) The 10th World Congress on Advances in Oncology, and 8th International Symposium on Molecular Medicine, Hersonissos, Crete, Greece, 13-15 October, 2005 – *Karel Smetana, Jr., Martin Chovanec, Barbora Dvorankova, Jan Boucek, Hans-Joachim Gabius – Comparative multiparametric analysis of the phenotype of FaDu cells with focus on*

Galectin expression – abstrakt publikovaný v International Journal of Molecular Medicine

h) 40th Annual Scientific Meeting of the European Society for Clinical Investigation, Prague, Czech Republic, 15 –18 March 2006 - *J. Boucek, J. Betka, J. Strohalm, D.Plocova, V. Subr, T.Mrkvan, K. Ulbrich, B. Rihova - In vitro efficacy of polymeric conjugates targeted by anti-EGFR monoclonal antibody or by human polyclonal immunoglobulin IgG* – přednáška

i) Dny průtokové cytometrie na Homolce, Praha, Česká republika, 3.-4.4.2006 - *J.Bouček, J.Betka, M.Chovanec,M.Kuchař, B.Říhová, M.Hladíková, T.Eckschlager - Cytometrické vyšetření u nádorů hlavy a krku,* přednáška

j) 69. Kongres České společnosti otorinolaryngologie a chirurgie hlavy a krku, Plzeň, Česká republika, 1.6. – 3.6. 2006 - *J. Bouček, J. Betka, M. Chovanec, M. Kuchař, T. Mrkvan, M. Hladíková, T. Eckschlager, B. Říhová - Regulační T lymfocyty u nádorů hlavy a krku* – poster

k) 3rd World Congress of International Federation of Head & Neck Oncologic Societies, Prague, Czech republic**,** June 27 – July 1, 2006 - *J. Boucek, J. Betka, J. Strohalm, D.Plocova, V. Subr, T.Mrkvan, K. Ulbrich, B. Rihova - Targeted polymeric anticancer drugs based on HPMA in therapy of HNSCC* – poster

l) 1st Joint Meeting of European National Societies of Immunology Under the auspices of EFIS, 16th European Congress of Immunology – ECI, 6. - 9. 9. 2006, Paris, France - *J. Boucek, J. Betka, J. Strohalm, D.Plocova, V. Subr, T.Mrkvan, K. Ulbrich, B. Rihova - In vitro therapy of the head and neck squamous cell carcinoma by targeted polymeric conjugates* - poster

m) Czech-Scottish Conference on Nanomedicine, 24. – 26. 9. 2006 – Praha, Česká republika – Jan *Bouček, Jan Betka, Tomáš Mrkvan, Ondřej Hovorka, Jiří Strohalm, Dana Plocová, Vladimír Šubr, Karel Ulbrich, Blanka Říhová – In vitro activity of HPMA-based conjugates targeted against EGFR* – přednáška

n) 11th World Congress on Advances in Oncology and 9th International Symposium on Molecular Medicine, October 12-14, 2006, Creta Maris Hotel, Hersonissos, Crete, Greece - *J. Boucek, J. Betka, M. Chovanec, M. Kuchar, T. Mrkvan, M. Hladikova, T. Eckschlager, B. Rihova - Regulatory T cells in patients with head and neck cancer* – přijatý abstract

o) Tumor Immunology: An Integrated Perspective, November 29 - December 2, 2006
InterContinental Miami Hotel, Miami, Florida, USA - *J. Boucek, J. Betka, J. Strohalm, D.Plocova, V. Subr, T.Mrkvan, O.Hovorka, K. Ulbrich, B. Rihova - ARE POLYMERIC CONJUGATES TARGETED TO EGFR INTERNALISIED BY RECEPTOR MEDIATED ENDOCYTOSIS?* – poster

p) II.dny diagnostické, prediktivní a experimentální onkologie, Olomouc, 7. – 9.12.2006 – *Eckschlager T., Bouček J., Betka J., Chovanec M., Kuchař M., Říhová B., Hladíková M. – Vyšetření lymfocytárních subpopulací u nádorů hlavy a krku – možnost predikce* – přednáška

q) International Symposium on Polymer Therapeutics (ISPT-07), February 19 – 21, 2007, Institute of Chemistry and Biochemistry Freie Universitat Berlin, Germany – *Blanka Rihova, Milada Sirova, Jan Boucek, Tomas Mrkvan, Ondrej Hovorka, Tomas Etrych, Vladimir Subr, Jiri Strohalm, Dana Plocova, Karel Ulbrich – HPMA based macromolecular therapeutics* - poster

r) New Targets and Delivery Systems for Cancer Diagnosis and Treatment, March 5-7, 2007, Hotel Del Coronado, San Diego, California, USA – *T.Mrkvan, J.Boucek, J.Strohalm, D.Plocova, K.Ulbrich, B.Rihova – Targeting Tumors Overexpressing EGFRs by Polymeric Nanotherapeutics Armed with Specific Monoclonal Antibodies and Doxorubicin* – přednáška

s) 6th European Congress of Oto-Rhino-Laryngology Head and Neck Surgery, Congress Center Hofburg, Vienna, Austria, June 30th - July 4th, 2007 - *J. Boucek, J. Betka, J. Strohalm, D.Plocova, V. Subr, T.Mrkvan, O.Hovorka, K. Ulbrich, B. Rihova - In vitro activity of HPMA-based targeted conjugates against HNSCC* – přijatý abstract

t) 12th World Congress on Advances in Oncology and 10th International Symposium on Molecular Medicine and Cancer Chemoprevention Symposium, October 11-13, 2007, Creta Maris Hotel, Hersonissos, Crete, Greece - *J. Boucek, M. Chovanec, M. Kuchar, T. Mrkvan, V. Boucek, M. Hladikova, J. Betka, T. Eckschlager, B. Rihova - Head and neck squamous cell carcinoma and regulatory T cells* – **vyžádaná přednáška**

u) 35th Congress of the International Society for Oncodevelopmental Biology and Medicine, Prague, Czech Republic, September 15th - 19th, 2007- *J. Boucek, T.Mrkvan, O.Hovorka, J. Strohalm, D.Plocova, V. Subr, J. Betka, K. Ulbrich, B. Rihova - ANTI-EGFR TARGETED POLYMERIC CONJUGATES AND MECHANISM OF THEIR INTERNALIZATION* – poster

v) Molecular Targets and Cancer Therapeutics, October 22 – 26, 2007, San Francisco, USA - *J. Boucek, T.Mrkvan, O.Hovorka, J. Strohalm, V. Subr, D.Plocova, J. Betka, K. Ulbrich, B. Rihova - In vitro efficacy of HPMA based conjugates containing doxorubicin and anti-EGFR mAb* – poster

w) X. Motolské onkologické dny - 3. 4. 2008, - *J. Bouček, M. Chovanec, M. Kuchař, T. Mrkvan, J. Betka, M. Hladíková, J. Betka, T. Eckschlager, B. Říhová - Treg a dlaždicobuněčné nádory hlavy a krku,* ,– **vyžádaná přednáška**

x) XII. Kongres mladých otorinolaryngologů – Jablonné nad Orlicí, 14. – 16. 5. 2008 - *J. Bouček, M. Chovanec, M. Kuchař, T. Mrkvan, J. Betka, M. Hladíková, J. Betka, T. Eckschlager, B. Říhová - Spinocelulární karcinomy hlavy a krku a regulace protinádorové imunity,* přednáška

y) 9. studentská vědecká konference 1.LFUK - 21. 5. 2008 - *J. Bouček, J. Betka, T. Eckschlager, B. Říhová - Význam regulačních T lymfocytů u spinocelulárních karcinomů hlavy a krku* – přednáška

z) Tumor Immunology: New Perspectives, December 2 - 5, 2008, Miami, Florida, USA - *J. Boucek, T.Mrkvan, O.Hovorka, J. Strohalm, V. Subr, D.Plocova, J. Betka, K. Ulbrich, B. Rihova - In Vitro Efficacy of HPMA Based Conjugates Containing Doxorubicin and anti-EGFR mAb in HNSCC* – poster

aa) 1st Meeting of the European Academy of ORL-HNS, June 27 - 30, 2009, Congress Center Rosengarten, Mannheim, GERMANY - *J. Boucek, M. Chovanec, M. Kuchar, T. Mrkvan, V. Boucek, M. Hladikova, J. Betka, T. Eckschlager, B. Rihova - Prognostic Value of Regulatory T cells for Early Recurrence of Head and Neck Squamous Cell Carcinoma* – poster (**vybrán jako: "candidate for the Poster Awards")**

bb) 73. kongres České společnosti otorinolaryngologie a chirurgie hlavy a krku, 16.-18. června 2010, Mikulov - *J. Bouček, T. Mrkvan, M. Chovanec, Jar.Betka, V. Bouček, M. Hladíková, Jan Betka, T. Eckschlager, B. Říhová - Význam imunokompetentních buněk pro diagnostiku nádorů v oblasti hlavy a krku* – přednáška

cc) Third International Symposium on Bone Conduction Hearing – Craniofacial Osseointegration, 22.-26.3.2011, Sarasota, USA - *Boucek, J., Cerny, L., Vokral, J., Skrivan, J. - BP-100 And Single-Sided Deaf Patients In Czech Republic* - poster

Vlastní publikační aktivita:

1. Regulatory T cells and their prognostic value for patients with squamous cell carcinoma of the head and neck.

   Boucek J, Mrkvan T, Chovanec M, Kuchar M, Betka J, Boucek V, Hladikova M, Betka J, Eckschlager T, Rihova B.

   J Cell Mol Med. 2010 Jan;14(1-2):426-33. Epub 2009 Jan 14.

   IF = 5,228

   Komentář:

Práce se zabývá imunologickými aspekty spinocelulárních karcinomů hlavy a krku. V době diagnózy bylo do studie zařazeno 112 pacientů s verifikovaným spinocelulárním karcinomem v oblasti hlavy a krku. Pořadí bylo náhodné, inkluzními kritérii byl věk, negativní onkologická a imunologická anamnéza.

Ze vzorků periferní krve bylo provedeno biochemické vyšetření (IgG, IgA, IgM, IgE, CRP, SCC, AAT, CEA, Cyfra 21-1), vyšetření krevního obrazu s diferenciálním počtem a vyšetření základních lymfocytárních subpopulací, včetně Treg (CD3+, CD4+, CD4+CD25+, CD8+, CD4+/CD8+, CD14+, CD19+, CD45+, CD56+, CD4+CD45RA, CD3-CD56+).

Studie prokázala u jednoho z největších souborů pacientů s HNSCC zabývajících se touto problematikou odchylky v mnoha parametrech imunitního systému. Změny se týkají absolutního počtu i procentuálního zastoupení CD8+ i CD4+ lymfocytů, počtu NK buněk, snížení hladiny B lymfocytů (CD19+).

Procento regulačních T lymfocytů je v periferní krvi pacientů s HNSCC signifikantně zvýšené oproti zdravým kontrolám. Za zásadní výsledek této práce považuji prokázání korelace zvýšeného počtu Treg v periferní krvi pacientů v době stanovení diagnózy a časné recidivy onemocnění (v průběhu 1. roku od ukončení léčby). Vstupní vyšetřování hladiny Treg by mohlo přispět k efektivnějšímu rozhodování při volbě terapie a následné dispenzarizaci pacientů po ukončení léčby.

2. Doxorubicin release is not a prerequisite for the in vitro cytotoxicity of HPMA-based pharmaceuticals: in vitro effect of extra drug-free GlyPheLeuGly sequences.

   Říhová B, Strohalm J, Hovorka O, Subr V, Etrych T, Chytil P, Pola R, Plocová D, Boucek J, Ulbrich K.

   J Control Release. 2008 Apr 21;127(2):110-20. Epub 2008 Jan 16.

   IF = 5,949

Komentář:

V práci jsou popsány výsledky systematické studie zabývající se významem typu postranního řetězce v HPMA polymerních léčivech vázajících doxorubicin. Na HPMA založená léčiva jsou velmi slibnou terapeutickou zbraní, v mnoha experimentech schopnou vyléčit až 100% léčených myší, za cenu minimálních nežádoucích účinků a zejména ve vhodném schématu umožňující vznik protektivní dlouhodobé imunologické paměti. Přes nepochybně vysokou účinnost v experimentu, nejsou ještě zcela objasněny některé mechanizmy účinku těchto terapeutik, zejména v souvislosti s jejich strukturou. V případě aminolyticky štěpitelných polymerních konjugátů je postranní oligopeptidický řetězec využíván k vazbě jak samotné nízkomolekulární cytostatické látky, tak k vazbě směrující struktury. Typ postranního řetězce byl již mnohokrát popsán, ale tato práce předkládá jako první systematické důkazy o vlastnostech jednotlivých postranních řetězců, zejména pak o výhodných vlastnostech nejčastěji používaného tetrapeptidu glycyl-fenylalanyl-leucyl-glycyl (Gly-Phe-Leu-Gly). Na různých lidských i myších nádorových liniích bylo prokázáno, že konjugáty obsahující volné postranní řetězce Gly-Phe-Leu-Gly vykazují vyšší cytotoxicitu ve srovnání s analogem s jinou sekvencí či bez volných postranních Gly-Phe-Leu-Gly sekvencí. Tato aktivita není ovlivěna charakterem terminálního zakončení postranního řetězce. Zároveň bylo prokázáno, že schopnost intracelulární akumulace konjugátu je v případě přítomnosti postranního Gly-Phe-Leu-Gly tetrapeptidu největší.

3. New HPMA copolymer-based drug carriers with covalently bound hydrophobic substituents for solid tumour targeting.

   Chytil P, Etrych T, Konák C, Sírová M, Mrkvan T, Boucek J, Říhová B, Ulbrich K.

   J Control Release. 2008 Apr 21;127(2):121-30. Epub 2008 Jan 30.

   IF = 5,949

Komentář:

Předložená práce shrnuje výsledky testování několika struktur HPMA konjugátů vázajících doxorubicin pomocí hydrolyticky degradabilní hydrazonové vazby, lišících se navzájem typem hydrofobních substituentů navázaných podél polymerního řetězce. HPMA konjugáty vázající doxorubicin hydrazovou vazbou prokazují v experimentech řadu skvělých vlastností. Podstatou hydrazonové vazby je její stabilita při pH kolem 7,4, tedy v krevním řečišti. Po internalizaci nádorovou buňkou se v endosomech, kde klesá pH k hodnotám kolem 5-6, vazba rozpadá, tedy dojde k uvolnění nízkomolekulárního cytostatika. S cílem dosáhnout vyšší účinnosti a lepších farmakokinetických charakteristik byla hydrazonová struktura polymerního léčiva modifikována navázáním hydrofobních podjednotek dodecylu, kyseliny olejové a cholesterolu. V závislosti na typu a množství hydrofobních podjednotek tvoří ve vodném prostředí tyto konjugáty polymerní micely nebo stabilní hydrofobní nanopartikule (vel. cca 13-37mm v průměru). *In vitro* účinnost byla testována na celé řadě lidských i myších nádorových linií, *in vivo* byla léčiva testována na myších s inokulovaným EL-4 myším T lymfomem. Nejlepší farmakologické vlastnosti vykazoval konjugát vázající cholesterolové postranní podjednotky, kdy nejdéle cirkuloval v krevní plazmě a byl akumulován v tkáni tumoru. V závislosti na dávce bylo možné dlouhodobě vyléčit až 100% testovaných myší.

4. Nucleostemin expression in squamous cell carcinoma of the head and neck.

   Cada Z, Boucek J, Dvoranková B, Chovanec M, Plzák J, Kodet R, Betka J, Pinot GL, Gabius HJ, Smetana K Jr.

   Anticancer Res. 2007 Sep-Oct;27(5A):3279-84.

   IF = 1,428

   Komentář:

Předložená práce přináší první data o expresi nukleosteminu –proteinu typicky exprimovaného v jadércích kmenových buňek normálního dlaždicobuněčného epitelu orofaryngu a hrtanu – v buňkách spinocelulárních karcinomů z oblasti hlavy a krku. Vysoké zastoupení nukleosteminu v jadérkách bylo již prokázáno u řady jiných nádorů. Zatím není úplně jasné, zda je exprese nukleosteminu vlastností samotných nádorových buněk anebo je indukována nádorovými stromálními buňkami. Zároveň není ještě objasněn ani funkční význam nukleosteminu, spekuluje se o jeho významu jako inhibitoru buněčné senescence.

V této práci byla exprese nukleosteminu korelována s diferenciačními a proliferačními znaky epitelových buněk, jako jsou keratin 10, beta-katenin, galektin-1 nebo Ki67. Imunohistochemicky byly vyšetřeny vzorky z normálního epitelu hrtanu a hypofaryngu, z dlaždicobuněčných karcinomů z těchto oblastí a buňky tkáňové linie FaDu, lidské modelové linie spinocelulárního karcinomu, jak v podmínkách *in vitro*, tak *in vivo* po inokulaci a růstu v nu/nu myších.

Nukleostemin bylo možné detekovat v jadérkách jak nádorových buněk, tak v buňkách proliferující bazální vrstvy i v terminálních diferencovaných suprabazálních vrstvách normálního epitelu. Nicméně, množství nukleostemin pozitivních jader bylo ale signifikantně vyšší v maligních buňkách (vzorky karcinomů, FaDu buňky propagované jak *in vitro*, tak *in vivo)*. Proto je možné zařadit přítomnost nukleosteminu v jadércích jako jeden z markerů diferenciace nádoru (více zastoupen u méně diferencovaných) a jeho biologické povahy (více u maligních či agresivních tumorů).

5. Marker profiling of normal keratinocytes identifies the stroma from squamous cell carcinoma of the oral cavity as a modulatory microenvironment in co-culture.

   Lacina L, Dvoránkova B, Smetana K Jr, Chovanec M, Plzák J, Tachezy R, Kideryová L, Kucerová L, Cada Z, Boucek J, Kodet R, André S, Gabius HJ.

   Int J Radiat Biol. 2007 Nov-Dec;83(11-12):837-48.

   IF = 1,842

Komentář:

V práci jsou shrnuty poznatky o významu nádorového mikroprostředí, prokázané zejména vzájemným ovlivňováním mezi nádorovými keratinocyty a stromálními fibroblasty.

Stroma mikroprostředí, které je tvořeno zejména fibroblasty může významně ovlivňovat fenotypické aspekty přítomných buněk, ať již se jedná o nádorové či normální keratinocyty. Doposud převládal názor, že dochází k ovlivňování zejména ze strany nádorových keratinocytů směrem k ostatním buněčným elementům mikroprostředí. Možnost opačného vztahu byla *in vitro* testována pomocí kokultivačních experimentů za použití stromálních fibroblastů získaných z prostředí dlaždicobuněčných nádorů z oblasti hlavy a krku a zdravých keratinocytů získaných z vlasových folikulů.

Imunohistochemické analýzy prokázaly fenotypické změny na zdravých keratinocytech kokultivovaných s nádorovými fibroblasty ve srovnání s kokultivací bez přítomnosti buněk pocházejících z nádoru. Normální keratinocyty začaly pod vlivem nádorových fibroblastů exprimovat keratin 8 a 19 a jadérkový protein nukleostemin, tedy znaky asociované z nádorovými buňkami, jejichž zvýšená exprese koreluje s horší prognózou.

Nejzajímavějším a zároveň prioritním výsledkem této práce bylo, že stromální nádorové fibroblasty jsou schopné ovlivnit fenotyp normálních epiteliálních buněk, pokud jsou kultivovány ve společném médiu bez možnosti vzájemného kontaktu.

6. Nuclear presence of adhesion-/growth-regulatory galectins in normal/malignant cells of squamous epithelial origin.

   Smetana K Jr, Dvoránková B, Chovanec M, Boucek J, Klíma J, Motlík J, Lensch M, Kaltner H, André S, Gabius HJ.

   Histochem Cell Biol. 2006 Jan;125(1-2):171-82. Epub 2005 Oct 28.

   IF = 3,021

   Komentář:

Mezi mechanismy regulace buněčných aktivit jako jsou růst a adheze či naopak migrace patří i interakce mezi lektiny (cukry vázající proteiny) a karbohydráty na buněčné membráně. Galektiny jsou endogenní lektiny, přítomné v jádře i v cytoplazmě a mohou se vázat na řadu intracelulárních struktur. Analýza expresního profilu galektinů a jejich vazebných míst u různých typů buněk a v různých stádiích diferenciace je imunohistochemický přístup umožňující definovat závislost lokalizace daného znaku s růstovou aktivitou.

Pomocí monoklonálních protilátek byly hodnoceny rozdíly u FaDu buněk (modelová linie lidského dlaždicobuněčného karcinomu) a mezi dlouhodobě kultivovanými lidskými a prasečími normálními epidermálními buňkami v expresi homodimerického galektinu 1, 2, 7 a chimérickému galektinu 3. Dále byla testována exprese vazebných míst pomocí biotinylovaných lektinů.

Exprese jednotlivých galektinů v jádře a v cytoplazmě se liší v závislosti na řadě faktorů, jako je například délka kultivace buněk. U FaDu buněk se lišila exprese jednotlivých sledovaných znaků u buněk v různých růstových fázích, galektin 1 a galektin 3 byly lokalizovány v jádře v subconfluentní růstové fázi, stejně tak jako v cytoplazmě, kde ale při tvorbě vícevrstevné vrstvy buněk vymizí. Galektin 2 je u subkonfluentních buněk přítomen v jadérku, u konfluentních buněk v cytoplazmě a na rozdíl od galektinu 1 i v mezibuněčných kontaktech. Maximum pozitivity galektinu 3 je navíc u konfluentních buněk v oblasti mezibuněčných kontaktů. Exprese galektinu 7 nebyla detekována, ve shodě s dříve publikovanými daty.

Detailní cytochemická analýza je předpokladem pro výběr vhodných proteinů k cílené modulaci genové exprese a předstupněm jejich funkční analýzy.

Přílohy:

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Regulatory T cells and their prognostic value for patients with squamous cell carcinoma of the head and neck

Jan Boucek [a, b, *], Tomas Mrkvan [b], Martin Chovanec [a, g, h], Martin Kuchar [a], Jaroslav Betka [a, b], Vladimir Boucek [f], Marie Hladikova [d], Jan Betka [a], Tomas Eckschlager [c], Blanka Rihova [b]

[a] Charles University, 1st Faculty of Medicine, Department of Otorhinolaryngology Head and Neck Surgery, University Hospital Motol, V Uvalu, Prague, Czech Republic
[b] Institute of Microbiology ASCR, v.v.i., Videnska, Prague, Czech Republic
[c] Department of Paediatric Haematology and Oncology, Charles University, V Uvalu, Prague, Czech Republic
[d] Department of Medical Informatics, 2nd Faculty of Medicine, Charles University, V Uvalu, Prague, Czech Republic
[f] Department of Haematology and Blood transfusion, Hospital of Rudolf and Stephania Benesov, Machova, Benesov, Czech Republic
[g] Institute of Anatomy, 1st Faculty of Medicine, Charles University, U Nemocnice, Prague, Czech Republic
[h] Center of Cell Therapy and Tissue Repair, 2nd Faculty of Medicine, Charles University, University Hospital Motol, V Uvalu, Prague, Czech Republic

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Abstract

Regulatory T cells (Treg) are important regulators of anti-cancer immune responses, and an increase in Treg frequency was observed in the blood of cancer patients. Blood samples from 112 patients with head and neck squamous cell carcinoma antigen (HNSCC) were obtained at the time of tumour diagnosis, and lymphocyte subpopulations ($CD3^+$; $CD3^-CD16^+CD56^+$; $CD4^+$; $CD8^+$; $CD19^+$; $CD4^+CD45RA^+$) with emphasis on Treg counts ($CD3^+CD4^+CD25^+$), complete blood count and tumour markers (squamous cell carcinoma [SCC]; CEA; α-1-antitrypsin [AAT]; Cyfra 21–1; C-reactive protein [CRP]) were analysed. The data were grouped according to TNM classification, and their significance for the course of the disease at an interval of 1 year after the end of the therapy was determined. The percentage of $CD8^+$ cells increased and the CD4/CD8 ratio decreased with tumour grade. The ratio of B lymphocytes decreased in patients with locoregional metastases (11.25% *versus* 9.22%). Treg (15.2%) and $CD4^+$ cells (45.3%) increased, while NK cells (11.8%) decreased in HNSCC patients compared to controls (9.0%, 38.1% and 15.8%, respectively). The data obtained at time of diagnosis were used to assess the significance of tumour markers (SCC, Cyfra 21–1 and AAT) for evaluation of prognosis. The erythrocyte counts (4.64×10^{12}/l *versus* 4.45×10^{12}/l) and haemoglobin levels (14.58 g/dl *versus* 14.05 g/dl) decreased, while Treg counts (8.91% *versus* 15.70%) increased in patients with early recurrence. Our results show that examination of these parameters could be helpful for prognostication in HNSCC patients and aid improvement of treatment strategy.

Keywords: regulatory T cells • head and neck squamous cell carcinoma • tumour markers • early recurrence • lymphocyte subpopulations

Introduction

Regulatory T lymphocytes (Treg) represent one of the most important mechanisms of peripheral immune tolerance, which is employed to safeguard any over-activations of the immune system. It has been shown that interleukin (IL)-2 is vital for growth and differentiation of Treg [1]. It is worth noting that surface expression of the IL-2 receptor α, CD25, is not unique for Treg, and that activated conventional T cells also express CD25. Nonetheless, Treg represent a major population within the $CD4^+CD25^{hi}$ T cells repertoire in healthy individuals.

Transcriptional factor forkhead box P3 (Foxp3) is absolutely essential for Treg development. It is also the 'master regulator' of their regulatory functions. Furthermore, high levels of Foxp3

*Correspondence to: Jan BOUCEK, MD,
Department of Otorhinolaryngology,
Head and Neck Surgery, 1st Faculty of Medicine,
Charles University, University Hospital Motol,
V Uvalu 84, 150 06 Prague 5, Czech Republic.
Tel.: +420224434301
Fax: +420224434319
E-mail: jboucek99@seznam.cz

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expression were found almost exclusively amidst the $CD4^+CD25^+$ compartment of thymocytes and peripheral Treg [2–6].

It is now known that Tregs play a critical role in the induction of tolerance to self-antigens, including those expressed by tumours. Recently, published findings have shown that immune regulation mediated by Treg, which is vital for preventing autoimmunity, represents a mechanism whereby the efficient antitumour response is inhibited [4,7].

It was described that Treg frequency is increased in the peripheral circulation of patients with different types of tumours, and their accumulation in the tumour microenvironment may be a negative prognostic factor for some types of malignancies [8–11]. An increase in the number of T-regulatory lymphocytes in the peripheral circulation and at the tumour site has also been shown to correlate with progression of head and neck squamous cell carcinoma (HNSCC) [12–14].

Not only does increase in T-regulatory cell number interfere with the antitumour immune response, but at the same time, Treg cells may be the main obstacle undermining anti-cancer immunotherapy and active vaccination [15, 16].

Although significant advances in the treatment regimen for patients with HNSCC have been documented within the last 20 years, regrettably, survival rates for this disease have not improved for many years [17].

Thus, the development of new molecular markers, which could help to describe the biological and immunological status of patients and predict disease progression, may help with selecting the appropriate treatment modalities for individual patients [18–21].

Patients and methods

HNSCC patients

All patients diagnosed at the Department of Otorhinolaryngology and Head and Neck Surgery, 1st Faculty of Medicine, Charles University, University Hospital Motol with HNSCC without any previous oncological treatment between 2004 and 2006, and who were willing to participate in the study and sign the informed consent, were included in the study (n = 112; 97 males; 15 females; a median age of 59 years; range of 23–87 years). Samples of peripheral blood were obtained from each patient at the time of diagnosis. No other selection criteria in addition to those given above were applied. Patients subsequently underwent therapy with curative intent. Details of patient characteristics are shown in Table 1.

Healthy volunteers – blood donors

Control samples of peripheral blood were obtained from 20 healthy volunteers (blood donors) comprising 12 males and 8 females with a median age of 58 years (range 23–68 years). All controls were examined at the Department of Hematology and Blood transfusion, Hospital of Rudolf and Stephania, Benesov.

Table 1 Patient characteristics

Age	**[Years]**
Median age	59
Range	23–87
Sex	[*n*]
Male	97
Female	15
Total	112
Tumour site	
Oropharynx – base of tongue	24 (21%)
Oropharynx – tonsillar region*	41 (37%)
Hypopharynx	13 (12%)
Larynx	19 (17%)
Others **	15 (13%)
Tumour differentiation	
Poor (G 3–4)	39 (35%)
Moderate (G 2)	39 (35%)
Well (G 1)	25 (22%)
Not determined	9 (8%)
Tumour stage	
T1	17 (15%)
T2	37 (33%)
T3	34 (30%)
T4	21 (19%)
Unstaged	3 (3%)
Nodal status	
N0	38 (34%)
N1	17 (15%)
N2	50 (45%)
N3	7 (6%)
M stage	
M0	112
M1	0
Unstaged	0
Therapy after blood draw	
Surgery	14 (13%)
Surgery + radiotherapy	63 (56%)
Radiotherapy	27 (24%)
Radiochemotherapy	8 (7%)

Continued

Table 1 Continued

Smoking history	
Non-smoker	18 (16%)
With history of smoking	94 (84%)
Active (still smoking)	52 (46%)
Former (denied smoking at time of diagnosis)	42 (38%)
Alcohol history	
Total abstinence	0 (0%)
Daily alcohol consumption	31 (28%)

*Oropharynx – tonsillar region: tumours involving tonsillar region alone or with spread to the tonsillar pillars, soft palate or posterior wall of oropharynx

**Others: heterogeneous group of tumour localization – 4× carcinoma of the nasopharynx, 3× carcinoma of the nasal cavity, 3× carcinoma of the paranasal sinuses, 3× metastatic carcinoma with unknown primary localization, 2× carcinoma of the external auditory canal

All participants signed the informed consent approved by the Ethics Committee of the 2nd Medical Faculty of Charles University and University Hospital Motol.

Flow cytometry

Samples of peripheral blood were analysed by flow cytometry (FACSCalibur, BD, San Jose, CA, USA) after lysis of erythrocytes by FACS Lysing Solution (BD, San Jose, CA, USA) and staining with antibody-fluorochrome conjugates. We strictly adhered to instructions in the manufacturer's protocol for respective reagents. Antibodies anti-CD45 FITC/CD14 PE (to correctly set the gates for lymphocytes), anti-CD3 FITC/CD19 PE, anti-CD3 FITC/ CD16CD56 PE, anti-CD4 FITC/ CD8 PE, anti-CD45RA FITC/anti-CD4 PE and anti-CD3 FITC/ CD4 PE/CD25 APC (Beckmann Coulter, Nyon, Switzerland) were used. A total of 10,000 cells in the lymphocyte gate were acquired for analysis and the data were analysed with CellQuest software. Results are expressed as the percentage of respective cell subpopulations of all lymphocytes.

Total blood count and biochemical and tumour makers were examined in the Department of Clinical Haematology and Institute for Clinical Biochemistry and Pathological Biochemistry, University Hospital Motol, according to standard protocols.

Statistical analysis

In order to analyse the relationship between the different categories studied, the data were evaluated using a frequency 2×2 table chi-square test with Danderar's correction. All numerical data were presented as mean $\pm$ S.D., and were analysed statistically using Student's t-test. The correlations between immunological parameters and early recurrence of disease were evaluated by nonparametric Spearman's coefficient. *P*-values of less than 0.05 were considered significant. SPSS Software version 10.1 was used for all statistical calculations.

Results

We examined the peripheral blood of 112 patients with HNSCC. Blood samples were taken before the commencement of antitumour therapy. We focused on evaluation of lymphocyte subpopulations ($CD3^+$; $CD3^-CD16^+CD56^+$; $CD4^+$; $CD8^+$; $CD19^+$; $CD4^+CD45RA^+$; $CD3^+CD4^+CD25^+$), complete blood count and several tumour markers (SCC; CEA; AAT; Cyfra 21–1; CRP).

The levels of Treg and other lymphocyte populations were compared between HNSCC patients and those of healthy blood donors.

The absolute number of $CD3^+$ lymphocytes in the group of HNSCC patients was 2.02×10^9/l $\pm$ 0.67. The percentage of circulating $CD3^+CD4^+CD25^+$ and $CD4^+$ (Fig. 1A and B) cells significantly increased (both $P < 0.01$) in patients with HNSCC (15.2% $\pm$ 8.9 and 45.3 $\pm$ 9.6, respectively) in comparison with values from the control group (9.0 $\pm$ 4.3 and 38.1 $\pm$ 5.9, respectively) at time of diagnosis. There was no significant difference ($P = 0.05$) in ratios of either total T lymphocytes ($CD3^+$; 72.1% *versus* 65.8%) or effector T lymphocytes ($CD8^+$; 28.0% *versus* 28.4%). On the other hand, naïve T lymphocytes ($CD4^+45RA^+$; 14.7% *versus* 18.0%), B lymphocytes ($CD3^-CD19^+$; 9.8% *versus* 11.1%) and NK cells decreased in HNSCC patients, but only the decrease of NK cells was statistically significant ($CD3^-CD16^+CD56^+$; 11.8% $\pm$ 6.5 *versus* 15.8% $\pm$ 6.8; $P < 0.05$) (Fig. 1C).

The study included patients with tumours localized in different regions of the head and neck (Table 1). Despite the fact that all patients showed uniformly increased levels of Treg, we were able to provide further evidence for differences within patient groups based on the localization of primary tumour (oropharynx – tonsillar region 16.2% $CD3^+CD4^+CD25^+$; oropharynx – base of the tongue 15.2% $CD3^+CD4^+CD25^+$; hypopharynx 15.2% $CD3^+CD4^+CD25^+$; larynx 15.0% $CD3^+CD4^+CD25^+$; other localizations 12.9% $CD3^+CD4^+CD25^+$). The differences between patients with tumours of the oropharynx – base of tongue and hypopharynx were statistically significant in several variables. The levels of tumour marker α-1-antitrypsin (AAT; 1.45 $\pm$ 0.32 g/l *versus* 1.8 $\pm$ 0.35 g/l; $P = 0.008$) and levels of platelets (PLT; 225.4 $\pm$ 61.54 *versus* 317.1 $\pm$ 93.95 $\times 10^9$/l; $P = 0.047$) in patients with tumours of the hypopharynx were higher. (There was no statistically significant difference between T- and N stages, and tumour differentiation grading, but there was a difference in distribution by gender: no females presented with tumour of the oropharynx – base of tongue, while three females presented with tumour of the hypopharynx.)

Relevance of Treg levels in relation to the stage of TNM classification was also evaluated. Patient groups were divided according to the size of the primary tumour (T1 to T4 stage), and the spread of tumour to the regional lymphatic nodes (N stage; N° *versus* N^+) according to the standards of International Classification of Diseases for Oncology (ICD-O-3, 2000).

All stages (T1 – T4) were individually compared, and no significant differences in Treg were observed (14.77% *versus* 17.16% *versus* 13.91% *versus* 14.91%). There was however a statistically

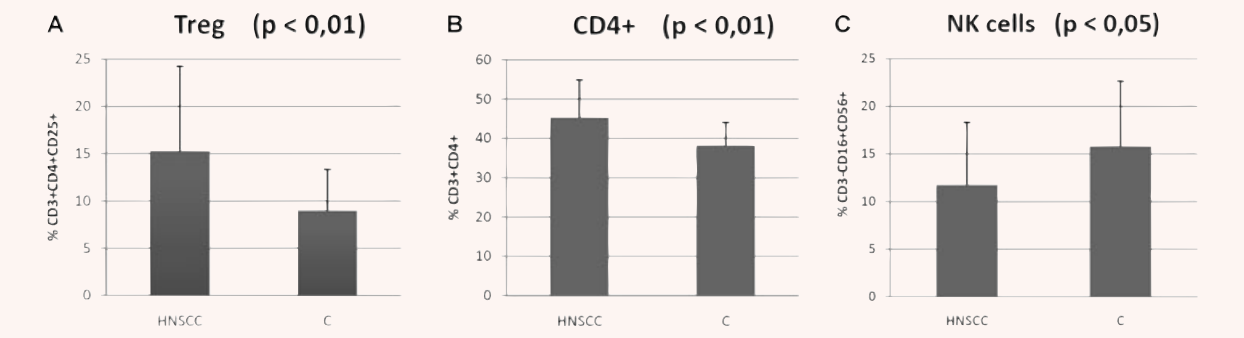

Fig. 1 Comparison of Treg and other lymphocyte subpopulations in patients with HNSCC (head and neck squamous cell carcinoma) with those of healthy blood donors (**C**). (**A**) – regulatory T lymphocytes ($CD3^+CD4^+CD25+$); (**B**) – Th cells ($CD3^+CD4^+$); (**C**) – natural killers (CD3- CD16+CD56+).

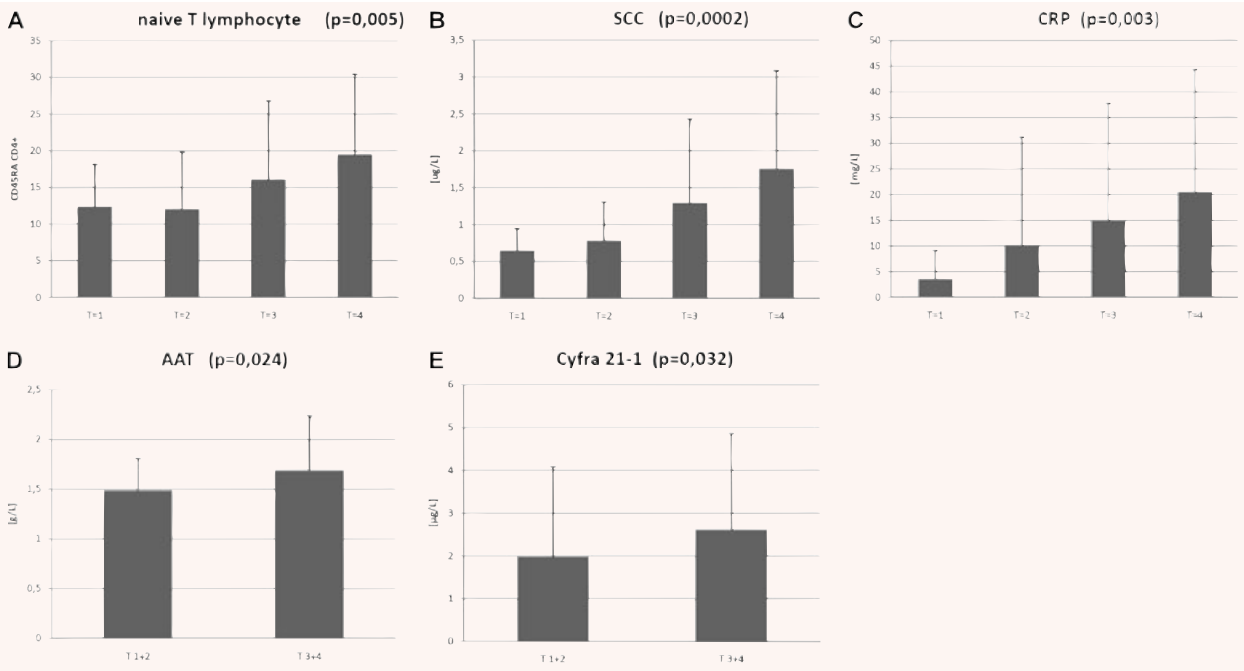

Fig. 2 Statistically significant parameters that are in a positive correlation with the size of the tumour (T stage). (**A**) – naïve T lymphocytes ($CD45RA^+CD4^+$); (**B**) – SCC = squamous cell carcinoma antigen; (**C**) – CRP = C-reactive protein; (**D**) – AAT = α-1-antitrypsin; (**E**) – Cyfra 21–1.

significant increase in the tumor marker SCC (SCC; 0.65 μg/l *versus* 0.78 μg/l *versus* 1.29 μg/l *versus* 1.76 μg/l; $P = 0.0002$); the ratio of naïve T lymphocytes (12.38% *versus* 12.05% *versus* 16.08% *versus* 19.49%) and the levels of C-reactive protein (CRP; 3.56 mg/l *versus* 10.18 mg/l *versus* 14.93 mg/l *versus* 20.49 mg/l; $P = 0.008$) (Fig. 2).

When we combined results for both T1 and T2 stages, and T3 plus T4 stages, and compared T1/2 *versus* T3/4, we found an increase of other two tumour markers in advanced stage patients, in particular, AAT (1.49 g/l *versus* 1.69 g/l; $P = 0.024$) and Cyfra-21–1 (1.99 μg/l *versus* 2.62 μg/l ; $P = 0.032$) (Fig. 2).

The levels of tumour marker Cyfra-21–1 and CRP were higher in the N^+ group than in the N0 group (1.39 μg/l *versus* 2.78 μg/l; $P = 0.00004$, respectively, 5.29 *versus* 16.18; $P = 0.023$). The percentage of B cells (CD3–19+) was significantly lower in the group of patients with locoregional metastases than in those patients with N0 stage disease (11.25% *versus* 9.22%; $P = 0.019$) (Fig. 3).

Levels of Treg were evaluated based on differentiation of tumour cells according to histological grading (G stage, G1 *versus* G2 *versus* G3+4). There were no significant differences in levels of Treg (14.85% *versus* 15.84% *versus* 14.25%). In other subgroups of lymphocytes, differences in levels of cytotoxic T lymphocytes

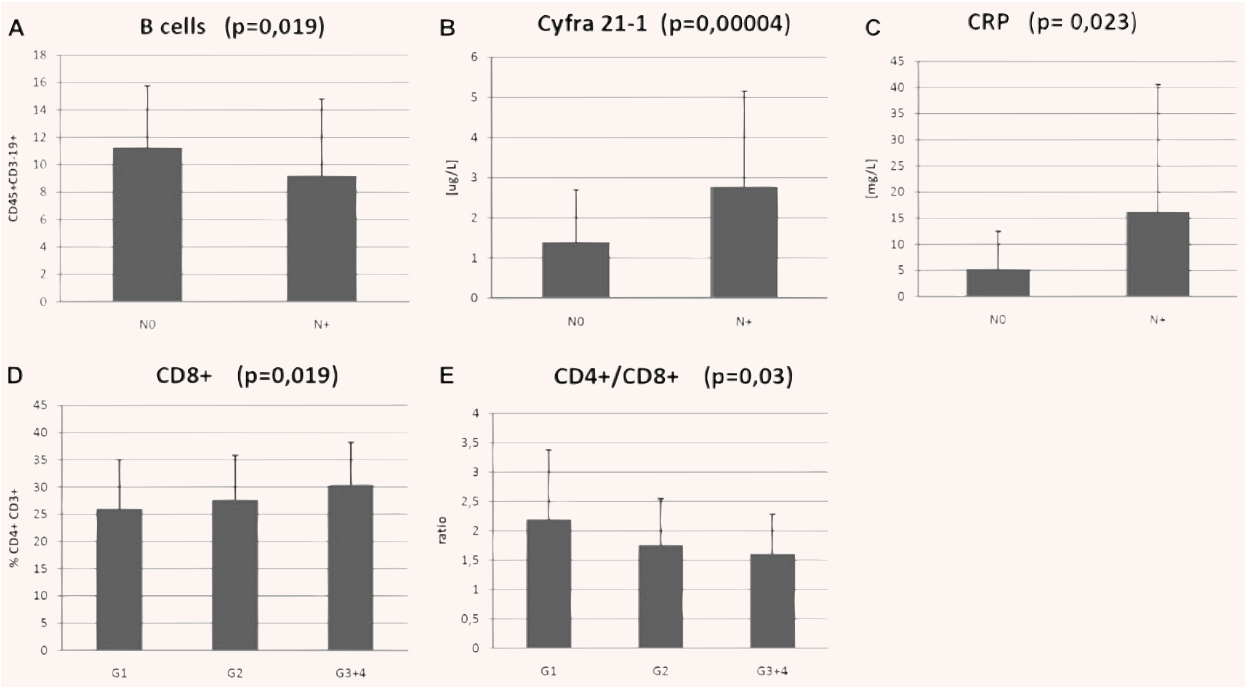

Fig. 3 Statistically significant parameters that are in a correlation with the spread of tumour to the regional lymphatic nodes (N stage) and with differentiation of tumour cells (Grade). (**A**) – B cells (CD45+CD3-CD19+); (**B**) – Cyfra 21–1; (**C**) – CRP = C-reactive protein; (**D**) – Tc cells ($CD3^+$ $CD8^+$); (**E**) – ratio $CD4^+/CD8^+$.

($CD8^+$; 26.04% *versus* 27.69% *versus* 30.41%; $P = 0.019$) and in CD4/CD8 ratio were observed (2.20 *versus* 1.76 *versus* 1.61; $P = 0.03$) (Fig. 3).

The group of patients with recurrent disease was compared with the group without evidence of the disease at an interval of 1 year after the end of the therapy. All of the following results were statistically significant: increase in levels of Treg (8.91% *versus* 15.70%; $P = 0.044$); increase in AAT (1.51 g/l *versus* 1.71 g/l; $P = 0.006$); decrease of erythrocyte count (4.64×10^{12}/l *versus* 4.45×10^{12}/l; $P = 0.038$) and increase in haemoglobin levels (14.58 g/dl *versus* 14.05 g/dl; $P = 0.022$) (Fig. 4).

Discussion

It has been published that clinical prognosis of oncological patients is correlated with numerous changes in the peripheral blood. Furthermore, it has been proven that the poor prognosis of HNSCC patients is associated with cancer cachexia, T status, increased C-reactive protein and decreased haemoglobin levels [22]. In agreement with these findings, we have demonstrated a statistically significant difference in haemoglobin level and erythrocyte count between the group of patients without evidence of disease, and the group with cancer relapse.

The advantages of examining tumour markers for improvement of the clinical management of HNSCC have been discussed for many years, yet none of these markers have been found to be exclusively predictive. Hepatocyte growth factor, which correlates highly with tumour progression, and may be a strong predictor of HNSCC recurrence, has recently been reported as being quite promising [23]. It has been published that combined analysis of SCC and CEA leads to both a markedly increased sensitivity at primary diagnosis, and as a predictor of tumour relapse [24]. Our study demonstrates that serum levels of SCC, CRP, Cyfra 21–1 and AAT correlate with T stage of disease, and that serum levels of Cyfra 21–1 and CRP correlate with N stage.

Treg ($CD4^+CD25^+FoxP3^+$), which are a subset of $CD4^+$ cells, have considerable importance within the immunological homeostatic network. These cells possess suppressive activity against $CD8^+$ effector and $CD4^+$ helper T cells. The mechanism of suppression is still unclear and the subject of controversial debate [25–27]. An increase in T-regulatory cells in the peripheral circulation and at the tumour site was previously reported in patients with HNSCC, and these results seem to be in agreement with the majority of published data for other human cancers [8, 12, 28–30]. In addition, a positive association with infiltration by Treg and better locoregional control of the tumour or longer disease-free interval was also reported [31].

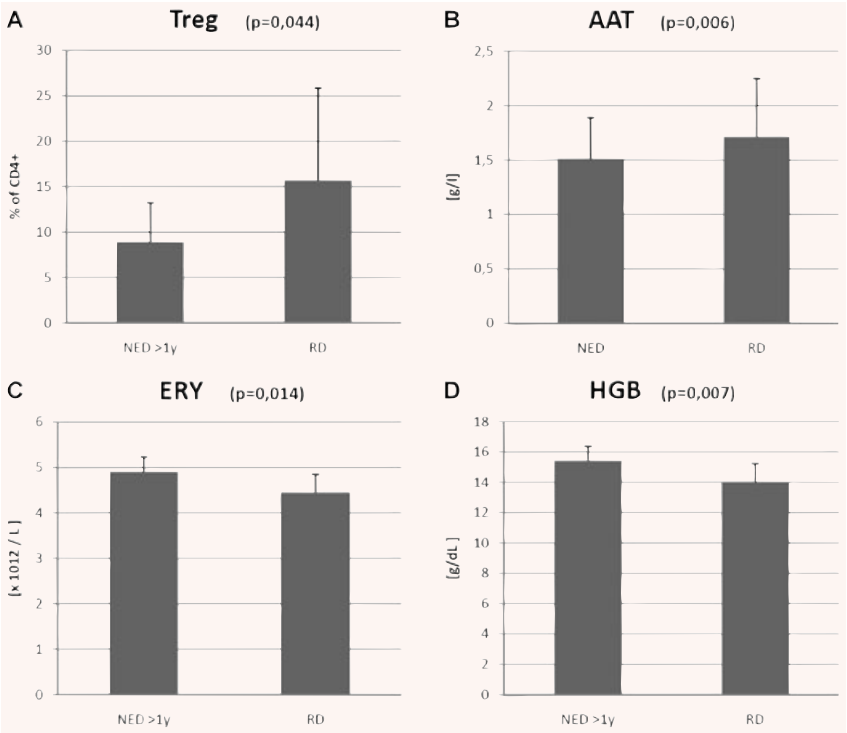

Fig. 4 Comparison between groups of patients with recurrent disease (RD) and those without evidence of disease (NED) over a follow-up interval longer than 1 year. (**A**) – regulatory T lymphocytes ($CD3^+CD4^+CD25+$); (**B**) – AAT = α-1-antitrypsin; (**C**) – ERY = count of erythrocyte; (**D**) – HBG = haemoglobin level.

The data for this study were collected over more than 3 years, and initially, there was no readily available monoclonal antibody against Foxp3. We have therefore analysed the $CD3^+CD4^+CD25^+$ cell population, which consists predominantly of regulatory T-cells.

Previous studies have indicated that patients with HNSCC have altered lymphocyte homeostasis, which persists for months or years after curative therapies [32, 33].

NK cells play one of the pivotal roles in antitumour immunity. Recently, it was published that NK cell numbers are reduced in the peripheral blood of cancer patients, and that a severe deficiency in circulating NK cells was related to the poor clinical outcome in HNSCC patients [34]. Our data confirmed these results. We found a significantly decreased proportion of NK cells in HNSCC patients compared with controls.

Other studies have reported that patients with HNSCC have significantly lower absolute numbers of $CD3^+$, $CD4^+$ and $CD8^+$ T cells, but no differences in the percentages of T-cell subsets between patients and controls were observed [33, 35]. We did not observe any significant differences either in the absolute number of $CD3^+$T cells or in percentages of T-cell subsets. Furthermore, Kuss *et al.* [32] described a decrease in absolute numbers of $CD3^+$ and $CD4^+$ ($P = 0.06$), and an increase in absolute numbers of $CD8^+$ ($P = 0.95$) in the peripheral circulation of patients with recurrent HNSCC within 2 years after therapy. Kim *et al.* suggested that such altered homeostasis in $CD8^+$ T cells in these patients is prevented as a consequence of cancer induced functional abnormalities and abnormal lymphocyte turnover [36]. Our data indicate that this alteration, which was first reported in patients who completed the course of therapy [32], could be inherent for HNSCC patients, as a significant increase of $CD8^+$ T cells in patients with recurrent HNSCC (compared to NED patients) was demonstrated as early as at the time of diagnosis.

Moreover, we found that the significant increase in $CD8^+$ subsets in patients directly correlated with the level of tumour cell differentiation, *i.e.* histological grading (Grading, G1 *versus* G2 *versus* G3+4). Similarly, a decrease in the CD4/CD8 ratio was found.

However, the exact relationship of elevated $CD8^+$ cells (and changes in CD4/CD8 homeostasis, respectively) with disease progression is still not clear, and should be investigated in future studies.

Excessive peritumoural infiltration of B cells ($CD19^+$) has been recently described, and a higher percentage of $CD19^+$ cells were predictive of poor survival in patients with ovarian carcinoma [37]. We identified a lower level of B cells ($CD3^-CD19^+$) in the group of patients with locoregional metastases compared with patients in the N0 stage. The explanation for this could be that the B cells are chemotactically attracted to the microenvironment of the tumour and metastases [38].

Tumour production of growth factors and the immunological reaction within the tumour microenvironment causes mobilization of precursor cells with subsequent migration to the periphery and tumour site [39]. In agreement with the previously published data we found a statistically significant positive correlation with the T stage and percentage of naïve T lymphocytes.

In this study, we focused on the quantity of Treg in the peripheral blood of patients with head and neck cancer, and compared

this data with controls in an effort to assess their prognostic importance for early recurrence of disease.

First, an increased percentage of circulating $CD4^+CD25^+$ T cells in the peripheral blood in patients with HNSCC was observed, which is in agreement with previously reported data [8, 9, 12–14]. All hitherto published data have shown that the total amount of Treg in the peripheral circulation of HNSCC is two-fold higher than in controls, although the exact numbers are slightly different among the different laboratories [12, 13].

In the 2-year long follow-up interval, we compared the group of patients with early recurrence of disease with the disease-free group. We found a striking difference in the levels of Treg at the time of primary diagnosis between patients in remission and in recurrence. The levels of Treg in the peripheral blood correlate with a higher probability of early recurrence of HNSCC. This finding helped us to select patients eligible for more extensive therapy and more meticulous follow-up.

In conclusion, it was observed that the percentage of CD8+ cells increased and CD4/CD8 ratio decreased with tumour grade. B lymphocyte proportion decreased in patients with locoregional metastases (N^+ stage). Treg ($CD3^+CD4^+CD25^+$) and $CD4^+$ cells increased, while NK cells decreased in HNSCC patients compared to healthy controls. Based on the data obtained at the time of primary diagnoses, we assessed the significance of tumour markers (SCC, Cyfra 21–1 and AAT) for evaluation of prognosis of HNSCC patients. The erythrocyte count and haemoglobin level decreased, while the Treg increased in the group of patients with early recurrence of the disease. One may speculate that erythrocyte count, haemoglobin level and regulatory T-cell proportion may be useful as predictive factors in HNSCC.

Acknowledgements

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References

1. **Groux H.** Type 1 T-regulatory cells: their role in the control of immune responses. *Transplantation*. 2003; 75: 8S–12S.
2. **Carrier Y, Yuan J, Kuchroo VK, *et al*.** Th3 cells in peripheral tolerance. I. Induction of Foxp3-positive regulatory T cells by Th3 cells derived from TGF-beta T cell-transgenic mice. *J Immunol*. 2007; 178: 179–85.
3. **Ladi E, Yin X, Chtanova T, *et al*.** Thymic microenvironments for T cell differentiation and selection. *Nature Immunol*. 2006; 7: 338–43.
4. **Randolph DA, Fathman CG.** CD4 + CD25+ regulatory T cells and their therapeutic potential. *Ann Rev Med*. 2006; 57: 381–402.
5. **Sakaguchi S.** Naturally arising Foxp3-expressing CD25+CD4+ regulatory T cells in immunological tolerance to self and non-self. *Nature Immunol*. 2005; 6: 345–52.
6. **Campbell DJ, Ziegler SF.** FOXP3 modifies the phenotypic and functional properties of regulatory T cells. *Nature Rev*. 2007; 7: 305–10.
7. **Gallimore A, Godkin A.** Regulatory T cells and tumour immunity – observations in mice and men. *Immunology*. 2008; 123: 157–63.
8. **Curiel TJ, Coukos G, Zou L, *et al*.** Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nature Med*. 2004; 10: 942–9.
9. **Wolf AM, Wolf D, Steurer M, *et al*.** Increase of regulatory T cells in the peripheral blood of cancer patients. *Clin Cancer Res*. 2003; 9: 606–12.
10. **Woo EY, Chu CS, Goletz TJ, *et al*.** Regulatory CD4(+)CD25(+) T cells in tumors from patients with early-stage non-small cell lung cancer and late-stage ovarian cancer. *Cancer Res*. 2001; 61: 4766–72.
11. **Strauss L, Bergmann C, Whiteside TL.** Functional and phenotypic characteristics of CD4+CD25highFoxp3+ Treg clones obtained from peripheral blood of patients with cancer. *Int J Cancer*. 2007; 121: 2473–83.
12. **Schaefer C, Kim GG, Albers A, *et al*.** Characteristics of CD4+CD25+ regulatory T cells in the peripheral circulation of patients with head and neck cancer. *Br J Cancer*. 2005; 92: 913–20.
13. **Strauss L, Bergmann C, Gooding W, *et al*.** The frequency and suppressor function of CD4+ CD25highFoxp3+ T cells in the circulation of patients with squamous cell carcinoma of the head and neck. *Clin Cancer Res*. 2007; 13: 6301–11.
14. **Alhamarneh O, Amarnath SM, Stafford ND, *et al*.** Regulatory T cells: what role do they play in antitumor immunity in patients with head and neck cancer? *Head Neck*. 2008; 30: 251–61.
15. **Whiteside TL.** Anti-tumor vaccines in head and neck cancer: targeting immune responses to the tumor. *Curr Cancer Drug Targets*. 2007; 7: 633–42.
16. **Karcher J, Dyckhoff G, Beckhove P, *et al*.** Antitumor vaccination in patients with head and neck squamous cell carcinomas with autologous virus-modified tumor cells. *Cancer Res*. 2004; 64: 8057–61.
17. **Forastiere A, Koch W, Trotti A, *et al*.** Head and neck cancer. *N Eng J Med*. 2001; 345: 1890–900.
18. **Plzak J, Betka J, Smetana K Jr, *et al*.** Galectin-3 – an emerging prognostic indicator in advanced head and neck carcinoma. *Eur J Cancer*. 2004; 40: 2324–30.
19. **Nestor M, Ekberg T, Dring J, *et al*.** Quantification of CD44v6 and EGFR expression in head and neck squamous cell carcinomas using a single-dose radioimmunoassay. *Tumour Biol*. 2007; 28: 253–63.
20. **Harper LJ, Piper K, Common J, *et al*.** Stem cell patterns in cell lines derived from head and neck squamous cell carcinoma. *J Oral Pathol Med*. 2007; 36: 594–603.
21. **Cada Z, Boucek J, Dvorankova B, *et al*.** Nucleostemin expression in squamous cell carcinoma of the head and neck. *Anticancer Res*. 2007; 27: 3279–84.

22. **Richey LM, George JR, Couch ME, *et al.*** Defining cancer cachexia in head and neck squamous cell carcinoma. *Clin Cancer Res*. 2007; 13: 6561–7.
23. **Kim CH, Lee JS, Kang SO, *et al.*** Serum hepatocyte growth factor as a marker of tumor activity in head and neck squamous cell carcinoma. *Oral Oncology*. 2007; 43: 1021–5.
24. **Wollenberg B, Jan V, Schmit UM, *et al.*** Cyfra 21–1 is not superior to SCC antigen and CEA in head and neck squamous cell cancer. *Anticancer Res*. 1996; 16: 3117–24.
25. **Scheffold A, Murphy KM, Hofer T.** Competition for cytokines: T(reg) cells take all. *Nature Immunol*. 2007; 8: 1285–7.
26. **Deaglio S, Dwyer KM, Gao W, *et al.*** Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression. *J Exp Med*. 2007; 204: 1257–65.
27. **Bopp T, Jonuleit H, Schmitt E.** Regulatory T cells–the renaissance of the suppressor T cells. *Ann Med*. 2007; 39: 322–34.
28. **Liyanage UK, Moore TT, Joo HG, *et al.*** Prevalence of regulatory T cells is increased in peripheral blood and tumor microenvironment of patients with pancreas or breast adenocarcinoma. *J Immunol*. 2002; 169: 2756–61.
29. **Albers AE, Ferris RL, Kim GG, *et al.*** Immune responses to p53 in patients with cancer: enrichment in tetramer+ p53 peptide-specific T cells and regulatory T cells at tumor sites. *Cancer Immunol Immunother*. 2005; 54: 1072– 81.
30. **Shevach EM.** Regulatory/suppressor T cells in health and disease. *Arthritis Rheum*. 2004; 50: 2721–4.
31. **Badoual C, Hans S, Rodriguez J, *et al.*** Prognostic value of tumor-infiltrating CD4+ T-cell subpopulations in head and neck cancers. *Clin Cancer Res*. 2006; 12: 465–72.
32. **Kuss I, Hathaway B, Ferris RL, *et al.*** Decreased absolute counts of T lymphocyte subsets and their relation to disease in squamous cell carcinoma of the head and neck. *Clin Cancer Res*. 2004; 10: 3755–62.
33. **Kuss I, Hathaway B, Ferris RL, *et al.*** Imbalance in absolute counts of T lymphocyte subsets in patients with head and neck cancer and its relation to disease. *Adv Otorhinolaryngol*. 2005; 62: 161–72.
34. **Molling JW, Langius JA, Langendijk JA, *et al.*** Low levels of circulating invariant natural killer T cells predict poor clinical outcome in patients with head and neck squamous cell carcinoma. *J Clin Oncol*. 2007; 25: 862–8.
35. **Chikamatsu K, Sakakura K, Whiteside TL, *et al.*** Relationships between regulatory T cells and CD8+ effector populations in patients with squamous cell carcinoma of the head and neck. *Head Neck*. 2007; 29: 120–7.
36. **Kim JW, Tsukishiro T, Johnson JT, *et al.*** Expression of pro- and antiapoptotic proteins in circulating CD8+ T cells of patients with squamous cell carcinoma of the head and neck. *Clin Cancer Res*. 2004; 10: 5101–10.
37. **Dong HP, Elstrand MB, Holth A, *et al.*** NK- and B-cell infiltration correlates with worse outcome in metastatic ovarian carcinoma. *Am J Clin Pathol*. 2006; 125: 451–8.
38. **Palmesino E, Moepps B, Gierschik P, *et al.*** Differences in CXCR4-mediated signaling in B cells. *Immunobiology*. 2006; 211: 377–89.
39. **Lathers DM, Lubbers E, Beal NM, *et al.*** Cultures derived from peripheral blood CD34+ progenitor cells of head and neck cancer patients and from cord blood are functionally different. *Hum Immunol*. 1999; 60: 1207–15.

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Doxorubicin release is not a prerequisite for the *in vitro* cytotoxicity of HPMA-based pharmaceuticals: *In vitro* effect of extra drug-free GlyPheLeuGly sequences

B. Říhová [a,*], J. Strohalm [b], O. Hovorka [a], V. Šubr [b], T. Etrych [b], P. Chytil [b], R. Pola [b], D. Plocová [b], J. Bouček [a,c], K. Ulbrich [b]

[a] *Institute of Microbiology, AS CR, v.v.i., Vídeňská 1083, 142 20 Prague 4, Czech Republic*
[b] *Institute of Macromolecular Chemistry, AS CR, v.v.i., Heyrovsky sq. 2, 162 06, Prague 6, Czech Republic*
[c] *Department of Otorhinolaryngology, Head and Neck Surgery, The First Medical Faculty, Charles University, University Hospital Motol, V Úvalu 84, 150 06, Prague 5, Czech Republic*

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Abstract

A systematic study was designed to elucidate differences in cytostatic activity *in vitro* between HPMA-based doxorubicin conjugates synthesized using different polymerization techniques and differing in peptidyl side chain. A polymer–drug conjugate containing doxorubicin (DOX) bound to HPMA copolymer backbone through the enzymaticaly non-cleavable sequence GlyGly shows low but significant cytotoxicity *in vitro* in seven cancer cell lines of mouse (EL4, 38C13, 3T3, BCL1) and human (SW620, Raji, Jurkat) origin. The low cytotoxicity can be considerably increased by the presence of additional drug-free GlyPheLeuGly side chains. P1 conjugate, i.e. non-targeted HPMA copolymer bearing doxorubicin bound via a biodegradable GlyPheLeuGly sequence, synthesized by direct copolymerization of HPMA with monomeric doxorubicin and thus without additional drug-free GlyPheLeuGly sequences is less effective compared to PK1 synthesized by polymer analogous reaction and thus containing extra drug-free GlyPheLeuGly sequences. Significant activity-enhancing effect was not seen with other amino acid/oligopeptide sequences (e.g., Gly or GlyGly). The activity-enhancing effect of GlyPheLeuGly sequences is more obvious in the conjugate containing doxorubicin bound to HPMA through GlyGly sequence. Derivatization of the terminal carboxyl group of the extra GlyPheLeuGly side chains (amide, *N*-substituted amide, free carboxyl) does not significantly influence the cytotoxicity of the conjugates. The presence of the GlyPheLeuGly sequence in the conjugate structure increases its rate of intracellular accumulation. Normal cells (Balb/c splenocytes) accumulate less polymer–doxorubicin conjugate compared to cancer cells (T cell lymphoma EL4, B cell lymphoma Raji and T cell leukemia JURKAT).

Keywords: HPMA; Apoptosis; Necrosis; Cancer cell line; GlyGly; GlyPheLeuGly; Extra side chain

1. Introduction

For many years we and others believed that the release of doxorubicin from its polymeric carrier based on *N*-(2-hydroxypropyl)methacrylamide (HPMA) is a prerequisite for the pharmacological activity of the conjugate [1,2]. The drugs were covalently linked to a water-soluble HPMA copolymer carrier either through proteolytically cleavable bond between an oligopeptidic spacer and the drug [3] or through a hydrolytically cleavable bond [4,5]. Proteolytical cleavability has been proven by bovine spleen cathepsins B, D, L, and H, and their mixture [6], chymotrypsin [7], by rat liver lysosomal enzymes prepared as tritosomes and by rat visceral yolk sacs *in vitro* [8,9]. Duncan [10] and Rejmanová et al. [11] reported a significant release of drug model, *p*-nitroaniline (NAp), from HPMA copolymers containing GlyPheLeuGlyNAp, GlyPheTyrAlaNAp, GlyPheLeuGlyPheNAp, GlyGlyPheLeuGlyPheNAp, AlaGlyValPheNAp, GlyGlyPhePheNAp, GlyGlyPheTyrNAp, AcapPheNAp and GlyLeuPheNAp. Říhová and Kopeček [1] were the first to

* Corresponding author. Tel.: +420 24106 2345.
E-mail address: rihova@biomed.cas.cz (B. Říhová).

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compare the efficacy of HPMA copolymer carrier containing a drug, daunomycin, bound to the polymeric backbone either through enzymatically non-cleavable sequence GlyGly or enzymatically cleavable sequence GlyPheLeuGly. Polyclonal antithymocyte (ATS) antibody was used as a targeting moiety. The efficacy of the targeting was tested as an inhibition of antibody response determined by direct counting of antibody-releasing splenocytes. Both *in vitro* cytotoxicity and *in vivo* antibody-inhibition efficacy were higher in conjugates containing daunomycin bound to the HPMA carrier through GlyPheLeuGly sequence. The result was confirmed *in vitro* and *in vivo* using a cancer model of mouse leukemia L1210 and human lymphoblastoid leukemia CCRF, Walker sarcoma or B16 melanoma [12,13]. Small molecules as carbohydrates, i.e. galactosamine and fucosylamine [14] or melanocyte stimulating hormone [13] were studied as targeting moieties. Říhová et al. [15] reported the same result with polymeric doxorubicin targeted with polyclonal anti-ATS or monoclonal anti-Thy 1.2 antibody. Once the impressive pharmacological activity of the conjugates containing doxorubicin bound through the cleavable tetrapeptide GlyPheLeuGly was confirmed *in vitro* and *in vivo* the conjugates with lower efficacy containing doxorubicin bound through proteolytically non-cleavable sequence GlyGly were tested only rarely. HPMA copolymer conjugate with doxorubicin bound to the polymeric carrier through tetrapeptide GlyPheLeuGly is also known as PK1 [16] or PK2 [17] and, as such it underwent Phase I and II clinical trials. Julyan et al. [17] reported that virtually all of the plasma doxorubicin remained polymer-bound during the period of study and no free doxorubicin or metabolites were detected in plasma samples. Urine analysis indicated that 76% of the doxorubicin administered was excreted during the first 24 h, mainly in polymer-bound form. Later on it was reported that there is no direct correlation between the rate of *in vitro* drug release from the conjugate and *in vitro* cytotoxicity of the HPMA copolymer conjugates and that the rate of the drug release is probably only one factor responsible for the pharmacological efficacy of conjugates based on HPMA [18]. Moreover, based on studies with intracellular localization of HPMA-based conjugates containing doxorubicin Hovorka et al. [19] hypothesized that the toxicity of such conjugates is a combination of a nucleotoxic effect of released doxorubicin and the effect of doxorubicin in a polymer-bound form directed against cell membranes. Damage to cytoplasmic, endosomal and lysosomal membranes induces disintegration of cell organization and metabolic collapse of the cell even before its apoptotic death can be induced.

In this paper we compared the cytotoxicity of PK1, i.e. non-targeted HPMA conjugate containing doxorubicin prepared by polymer analogous reaction [3] with P-1, i.e. non-targeted HPMA conjugate synthesized by direct copolymerization of HPMA with monomeric doxorubicin, i.e. comonomers containing tetrapeptide terminated in doxorubicin. Such conjugate does not contain extra drug-free GlyPheLeuGly side chains [20]. On the contrary, free radical precipitation copolymerization of HPMA monomer and methacryloylated (MA) peptides terminating in reactive *p*-nitrophenoxy groups (ONp) gives a polymeric intermediate to which drugs (DOX.HCl) containing a primary amino group can be subsequently bound by aminolysis in the presence of triethylamine in DMSO [21]. Such conjugates always contain additional GlyPheLeuGly side chains. Using PK1, P-1 and "non-cleavable" conjugate with GlyGly side chains terminating in doxorubicin but containing also drug-free GlyPheLeuGly side chains we address an important question what is the effect of such additional oligopeptide side chains remaining in the polymer structure after aminolytic conjugation reaction on cytostatic activity and accumulation of polymer–doxorubicin conjugates. Seven different cancer cell lines of human and mouse origin were used with different sensitivity to parent drug as well as to polymeric therapeutics.

2. Materials and methods

2.1. Chemicals

1-Aminopropan-2-ol, methacryloyl chloride, glycine, glycylglycine, glycyl-L-phenylalanine, L-leucylglycine, 4-nitrophenol (H-ONp), triethylamine (TEA), *N,N*-dimethylformamide (DMF), *N,N′*-dicyclohexylcarbodiimide (DCCI), 2,2′-azobisisobutyronitrile (AIBN), naphthalene-2,3-dicarboxaldehyde (NDA), dimethyl sulfoxide (DMSO) and doxorubicin hydrochloride (DOX.HCl) were from Fluka AG, Buchs (Switzerland). All other reagents and solvents (Sigma-Aldrich Ltd., Czech Republic) were of analytical grade. Solvents were dried and purified by conventional procedures and distilled before use.

2.2. Synthesis of monomers

N-(2-hydroxypropyl)methacrylamide (HPMA) was synthesized as described [17] with the exception of using Na_2CO_3 in place of $NAHCO_3$ as a scavenger of HCl. M.p. 70 °C; elemental analysis: calcd., C 58.72, H 9.15, N 9.78; found, C 58.98, H 9.18, N 9.82.

Methacryloylglycine (Ma-Gly-OH) was prepared by the reaction of 2.3 g (22 mmol) methacryloyl chloride dissolved in 3 ml dichloromethane with 1.5 g (20 mmol) glycine dissolved in 15 ml of aqueous solution of 1.68 g (42 mmol) NaOH. First, glycine was dissolved in the first half of NaOH solution (7.5 ml) and methacryloyl chloride solution was added simultaneously with the rest of NaOH solution (7.5 ml) in small portions under stirring and cooling to 0 °C. After 1 h stirring at 20 °C methylene chloride was removed, the reaction mixture was acidified to pH 2 with HCl and crude precipitated product was isolated by filtration and purified by crystallization from ethyl acetate. Yield was 80%, m.p. 106–108 °C, elemental analysis: calcd., C 50.35, H 6.34, N 9.78; found, C 50.38, H 6.33, N 9.70.

Methacryloylglycylglycine (Ma-GlyGly-OH) was prepared as described earlier [17]. M.p. 201–205 °C, elemental analysis: calcd., C 47.99, H 6.04, N 13.99; found, C 47.98, H 5.69, N 13.84. 4-Nitrophenyl ester Ma-GlyGly–ONp was prepared from Ma-GlyGly–OH and 4-nitrophenol using the DCCI method [17].

Methacryloylglycyl-DL-phenylalanyl-L-leucylglycine 4-nitrophenyl ester (Ma-GlyPheLeuGly-ONp) was prepared from methacryloylglycyl-DL-phenylalanyl-L-leucylglycine

(Ma-GlyPheLeuGly-OH) as previously described [18]. Characteristics of the nitrophenyl ester: m.p. = 134–136 °C, amino acid analysis: Gly:L-Phe:D-Phe:L-Leu:D-Leu = 2.06:0.54:0.46:1.00:0.02. HPLC showed two peaks of equal peak area at 14.41 min (L-Phe) and 14.71 min (D-Phe-containing peptide).

Methacryloylglycyl-DL-phenylalanyl-L-leucylglycyl 2-hydroxypropyl-1-amide (Ma-GlyPheLeuGly-AP) was prepared by aminolysis of 1 g (1.72 mmol) Ma-GlyPheLeuGly–ONp with 0.65 g (8.6 mmol) of 1-aminopropan-2-ol in 10 ml DMF. Reaction mixture was stirred for 1 h, DMF was removed by vacuum evaporation and crude product was crystallized from ethyl acetate and purified on column packed with Kieselgel 60 (Merck). Yield 76%, m.p. elemental analysis: calcd., C 60.33, H 7.60, N 13.53; found, C 59.61, H 7.35, N 13.14. HPLC (C18 RP column) showed 2 peaks of equal area (D-Phe and L-Phe isomers). Amino acid analysis: Gly:L-Phe:D-Phe:L-Leu:D-Leu = 2.03:0.55:0.46:1.00.

Methacryloylglycyl-DL-phenylalanyl-L-leucylglycyl amide of doxorubicin (Ma-GlyPheLeuGly-DOX) was prepared by aminolysis of 300 mg (0.52 mmol) Ma-GlyPheLeuGly–ONp with 300 mg (0.51 mmol) DOX.HCl carried out in 3 ml DMF containing an equivalent of TEA (60 mg) using a procedure described in [17]. HPLC analysis (C18 RP column) showed 2 peaks of equal area, amino acid analysis: Gly:L-Phe:D-Phe:L-Leu:D-Leu = 2.07:0.54:0.45:1.00:0.006.

2.3. Synthesis of polymer–drug conjugates

HPMA copolymer with DOX bound via –GlyPheLeuGly– spacer (PK1) was prepared by copolymerization of HPMA with Ma-GlyPheLeuGly–ONp followed by aminolysis of the copolymer with DOX.HCl carried out in the presence of triethylamine in DMSO as described earlier [17].

HPMA copolymer with DOX bound via –GlyPheLeuGly– spacer to the polymer backbone (P-1) was prepared by direct radical copolymerization of 330 mg (2.3 mmol) HPMA with 49 mg (0.05 mmol) Ma-GlyPheLeuGly–DOX in 3 ml of methanol. Monomers and 22.3 mg (0.136 nmol) AIBN were dissolved in methanol, placed into polymerization ampoule, bubbled through with oxygen-free nitrogen and the ampoule was sealed. Polymerization carried out at 60 °C was stopped after 16 h by precipitation of the polymer into acetone. The polymer was reprecipitated from methanol into acetone, isolated by filtration and dried in vacuum. Crude copolymer was fractionated on Sephacryl S-300 column using saline solution (0.15 M NaCl) as eluent. Low-molecular-weight fractions were removed to obtain a copolymer with narrow distribution of molecular weights. Final copolymer was desalinated on Sephadex G-25 column and lyophilized.

HPMA copolymer containing DOX and glycine in its side chains (P-2) was prepared by radical copolymerization of 320 mg HPMA (2.23 mmol), 48.8 mg (0.05 mmol) Ma-GlyPheLeuGly–DOX and 10 mg Ma-Gly–OH (0.065 mmol) in 2.5 ml of methanol using 24.3 mg (0.148 mmol) AIBN as initiator of polymerization. Polymerization was carried out as described above, polymerization time was 14 h. Copolymer was isolated by precipitation into a mixture of acetone–diethylether (3:1) and purified and fractionated by gel filtration as described above.

HPMA copolymer containing DOX and diglycine groups in its side chains (P-3) was prepared analogously to the synthesis of P-2. 316 mg (2.2 mmol) HPMA, 48.8 mg (0.05 mmol) Ma-GlyPheLeuGly–DOX and 14 mg (0.067 mmol) Ma-GlyGly–OH dissolved in 2.5 ml of methanol were used for polymerization initiated with 24.3 mg (0.148 mmol) AIBN.

HPMA copolymer containing DOX and 2-hydroxypropyl-1-amide groups in its side chains (P-4) was also prepared analogously to the synthesis of P-2. 294 mg (2.05 mmol) HPMA, 48.8 mg (0.05 mmol) Ma-GlyPheLeuGly–DOX and 36 mg (0.068 mmol) Ma-GlyPheLeuGly–AP together with 24.3 mg (0.148 mmol) AIBN dissolved in 2.5 ml of methanol were used for polymerization.

Conjugates poly(HPMA–*co*-Ma-GlyPheLeuGly-DOX–*co*-Ma-GlyPheLeuGly-X) (X = AP, OH or NH_2) were prepared from polymer precursor poly(HPMA–*co*-Ma-GlyPheLeuGly-DOX–*co*-Ma-GlyPheLeuGly-ONp) by aminolysis with 1-aminopropan-2-ol (P-5), hydrolysis in mildly alkaline solution (P-6) or aminolysis with ammonium (P-7).

The polymer precursor poly(HPMA–*co*-Ma-GlyPheLeuGly-DOX–*co*-Ma-GlyPheLeuGly-ONp) was prepared by precipitation radical copolymerization of 0.65 g (4.55 mmol) HPMA, 98.6 mg (0.1 mmol) Ma-GlyPheLeuGly–DOX and 163.8 mg (0.35 mmol) Ma-GlyPheLeuGly–ONp initiated with 54 mg (0.33 mmol) AIBN and carried out in 8 ml acetone at 50 °C for 24 h. The copolymer was isolated by filtration, washed with acetone, reprecipitated from methanol into diethyl ether and dried under vacuum. It contained 2 mol% DOX and 5.7 mol% ONp groups.

Aminolysis of 100 mg of the polymer precursor with tenfold excess of 1-aminopropan-2-ol was carried out in 1 ml of DMF solution for 10 min. Polymer P-5 was isolated by precipitation into diethyl ether, purified by reprecipitation from methanol into diethyl ether, dissolved in water and lyophilized.

Hydrolysis of 100 mg of the polymer precursor was carried out in $Na_2B_4O_7$ solution at pH 8.2 for 24 h on a Radiometer Copenhagen pH-stat. Hydrolyzed polymer P-6 was purified on Sephadex PD-10 in water and lyophilized.

Polymer P-7 was prepared by aminolysis of 100 mg of the polymer precursor dissolved in DMF with gaseous ammonium bubbled through the solution for 1 min. Final polymer was precipitated into acetone, purified on Sephadex PD-10 in water and lyophilized.

HPMA copolymer with DOX bound via –GlyGly– spacer (P-8) was prepared by copolymerization of HPMA with Ma-GlyGly–ONp followed by aminolysis of the copolymer with DOX.HCL in DMSO accordingly to PK1. Briefly, 2 g (13.9 mmol) HPMA, 0.236 g (0.074 mmol) Ma-GlyGly–ONp and 0.107 g (6.5 mmol) AIBN were dissolved in 18 ml acetone and polymerized under nitrogen in a sealed ampoule for 24 h at 50 °C. The polymer was isolated by filtration. 0.05 g (0.086 mmol) DOX.HCL was used for aminolysis of 0.5 g (0.185 mmol) P-GlyGly–ONp copolymer carried out in the presence of 0.013 ml triethylamine dissolved in 3.5 ml DMSO. Polymer conjugate was isolated after 4 h stirring at room

temperature by precipitation into acetone and purified by gel filtration using Sephadex LH-20 column and methanol as eluent.

Conjugate poly(HPMA–*co*-Ma-GlyGly-DOX–*co*-Ma-Gly-PheLeuGly-OH) (P-9) was prepared by aminolysis of a copolymer poly(HPMA–*co*-Ma-GlyGly-ONp–*co*-Ma-GlyPhe-LeuGly-OH) with DOX.HCl in DMSO carried out in the presence of triethylamine. The copolymer was prepared by copolymerization of 1.51 g (10.53 mmol) HPMA, 247 mg (0.77 mmol) Ma-GlyGly–ONp and 245 mg (0.53 mmol) Ma-GlyPheLeuGly–OH in 17.55 ml of acetone, polymerization was initiated with AIBN (96 mg, 0.58 mmol) and carried out in a sealed ampoule under nitrogen at 50 °C for 23 h. The polymer (precursor) of M_w=24 300 contained 6.1 mol% ONp and 4.5 mol% –COOH groups. Attachment of DOX to the polymer precursor was carried out in DMSO in accordance with the method described earlier [17].

Basic characteristics of polymers are given in Table 1.

2.4. Fractionation, purification and characterization of polymer conjugates

Polymer–drug conjugates were freed of low-molecular-weight impurities (such as monomers, initiator and its degradation products, etc.) by gel filtration using a Sephadex LH-20 (Amersham Bioscience) column with methanol elution. Fractionation of copolymers was conducted on GPC column packed with Sephacryl S-300 (Amersham Bioscience) using 0.15 M aqueous NaCl solution as eluent. GPC column was equipped with RI and UV/VIS detectors to follow polymer and DOX concentration (DOX at 488 nm). Eluted solutions were desalinated before lyophilization on Sephadex G-25 column using distilled water as eluent.

Molecular weights (weight and number average) of polymers were determined by HPLC (Shimadzu Co.) equipped with RI, UV/VIS and light scattering (EOS 8, Wyatt Technology Corp., USA) detectors. Superose™ 12 or Superose™ 6 columns, 0.3 M acetate buffer pH 6.5 as an eluent and flow rate 0.5 ml/min were used for analysis. All copolymers were tested for the content of free or monomeric drug using HPLC or combined extraction/HPLC analysis [19]. Less than 0.2% free or monomeric DOX was found in the products. Content of polymer-bound DOX was determined by UV/VIS spectrophotometry at 488 nm using molar extinction coefficient ε=11 500 g mol^{-1} l^{-1}. A list of conjugates used for biological evaluation is given in Table 1.

HPLC analysis of monomers was conducted on HPLC analyzer LDC Analytical, U.S.A. using reversed-phase column Tessek SGXC18, (125×4 mm), UV detection at 230 nm or 488 nm respectively, solvent methanol–water, gradient 50–100% methanol, and flow rate 0.5 ml/min.

Monomers with DOX were characterized by HPLC Shimadzu equipped with Chromolith RP-18e column (Merck) using gradient 10–90% methanol in water containing 0.1% TFA (trifluoroacetic acid), flow rate 1 ml/min.

Amino acid analysis: The ratio of amino acids in monomers, their optical purity and the content of glycine/diglycine or –GlyPheLeuGly– in the conjugates were determined by amino acid analysis with precolumn NDA derivatization on analyzer LDC Analytical, USA, using the reversed-phase column Tessek SGX C18, 250×4 mm, as described [17].

2.5. Cancer cell lines

Seven different cell lines of mouse (T cell lymphoma EL4; B cell lymphoma 38C13; fibrosarcoma 3T3; B cell leukemia BCL1) or human (metastazing colorectal carcinoma SW 620; B cell lymphoma Raji; T cell leukemia Jurkat) origin and normal Balb/c splenocytes as control representing diverse types of common experimental tumors were used for *in vitro* studies. All cell lines were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). Cancer cell line SW 620 was cultivated in RPMI 1640 medium with extra L-glutamine

Table 1
Characteristics of HPMA copolymers[a]

Polymer	Structure	M_w	M_n	DOX content wt.%	Content of free DOX wt.%[b]	ONp/COOH/AP content, mol%
PK1	P-G-F-L-G-DOX	24 800	17 000	5.1	0.3±0.02	3.0[c]
P-1	P-G-F-L-G-DOX	40 000	28 600	6.2	0	0
P-2	P-G-F-L-G-DOX\G-OH	55 000	42 300	6.5	0	2.2
P-3	P-G-F-L-G-DOX\G-G-OH	59 000	36 900	6.2	0	2.4
P-4	P-G-F-L-G-DOX\G-F-L-G-AP	40 000	28 600	6.4	0	1.9
P-5	P-G-F-L-G-DOX\G-F-L-G-AP	45 000	30 800	6.1	0	5.6[d]
P-6	P-G-F-L-G-DOX\G-F-L-G-OH	45 000	30 800	5.6	0	5.7[d]
P-7	P-G-F-L-G-DOX\G-F-L-G-NH_2	45 000	30 800	5.8	0	5.7[d]
P-8	P-G-G-DOX	21 200	13 200	7.5	0.04±0.01	3.0[e]
P-9	P-G-G-DOX\G-F-L-G-OH	39 500	22 000	5.2	0.05±0.02	4.6

P in a polymer structure means HPMA copolymer chain.
[a]One letter symbols for amino acid residues were used.
[b]Related to total DOX content.
[c]Molar content of G-F-L-G sequences terminating in COOH and/or AP groups, AP-2-hydroxypropyl-1-amide group, COOH/AP ratio was not determined.
[d]Molar content of G-F-L-G sequences terminating either in AP, COOH or in NH_2 group.
[e]Molar content of G-G sequences terminating in COOH and/or AP groups.

(4 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% v/v fetal bovine serum. Raji and Jurkat cell lines were cultivated in RPMI 1640 medium with extra L-glutamine (4 mM), 2-mercaptoethanol (5×10^{-5} M), penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% v/v fetal bovine serum. BCL1 and 38C13 cell lines were cultivated in RPMI 1640 with extra L-glutamine (4 mM), 2-mercaptoethanol (5×10^{-5} M), HEPES (10 mM), sodium pyruvate (1.0 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% v/v fetal bovine serum. EL4 T cell lymphoma was cultivated in RPMI 1640 medium with extra L-glutamine (4 mM), sodium pyruvate (1.0 mM), 4.5 g/l glucose, penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% v/v fetal bovine serum. 3T3 cell line was cultivated in D-MEM medium with extra L-glutamine (4 mM), HEPES (10 mM), 4.5 g/l glucose, penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% v/v fetal bovine serum.

2.6. Cell cultures

NUNCLON 96-well flat-bottomed plates (NUNC) were seeded with 5×10^3/well EL4 cells, 2×10^3/well 38C13 cells, 2×10^4/well 3T3 cells, 5×10^3/well BCL1 cells, 1×10^4/well SW 620 cells, 1×10^4/well JURKAT cells and 1×10^4 RAJI cells. The plates were cultured in 5% CO_2 at 37 °C. ^{3}H-thymidine was added after 72 h of cultivation.

2.7. Cytostatic activity in vitro

Was assessed using ^{3}H-thymidine incorporation assay [5].

2.8. Fluorescence quantification

Intensity of fluorescence was measured on BD LSR II as fluorescence of doxorubicin (Ex 488 nm, Em 560 nm).

2.9. Detection of necrosis and apoptosis

The pattern of cell death was determined using mouse T cell lymphoma EL4 by simultaneous staining with Annexin V-Dyomics 643 (5 μl/100 μl tested sample), room temperature, 20 min; Apronnex CZ) and Hoechst 33258 (0.1 μg/100 μl tested sample, room temperature, 5 min; Invitrogen USA). The percentage of apoptotic/necrotic cells was measured on BD LSRII flow cytometer. Negative control (NC) represents EL4 T cell lymphoma incubated in fresh medium, as a positive control (PC) were taken EL4 cancer cells incubated 12 h with free, i.e. non-modified doxorubicin (10 μg/ml).

2.10. Intracellular accumulation

Cells were incubated with doxorubicin (Meji Seika Kaisha, Japan) or polymeric conjugates in mentioned concentrations. Adherent cells were detached with 0,02% EDTA solution and washed with PBS/BSA. Cells were immediately analyzed on BD LSRII flow cytometer (doxorubicin Ex 488 nm/Em 580/25, Hoechst 33342 Ex 405 nm/Em 450/50, DiD Ex 488 nm/Em 530/30).

3. Results

3.1. Synthesis of conjugates

HPMA copolymer PK1 containing DOX bound to the polymer chain with a GlyPheLeuGly spacer was prepared by aminolysis of a polymer precursor (containing ONp reactive groups) with DOX.HCl in DMSO containing TEA. Such synthetic procedure results in a copolymer containing in its side chains, except for GlyPheLeuGly–DOX sequences, also a small amount of GlyPheLeuGly–OH (formed by hydrolysis) and GlyPheLeuGly–AP (originated during termination of the reaction with 1-aminopropane-2-ol) sequences (AP=2-hydroxypropyl-1-amide). To avoid the presence of these additional sequences and to study their effect on biological properties of the copolymer, also copolymers without (P-1) or with a well-defined amount of side chains terminated in carboxylic (G–OH, G–G–OH) (copolymers P-2 to P-3) or AP groups (copolymer P-4) were synthesized. The copolymers were prepared by radical copolymerization of HPMA with respective comonomers (contrary to PK1, prepared by aminolysis of an ONp groups-containing polymer precursor as described in numerous papers dealing with PK1 copolymer). This allowed the synthesis of conjugates with precisely defined composition. The conjugates P-5 to P-7 differing in the structure of a substituent of carbonyl group situated at the end of the sequence were prepared for a detailed study of an effect of structure of –GlyPheLeuGly– oligopepide sequence on the biological activity of polymer conjugates. Simple amide group (NH_2), hydrophilic *N*-substituted amide group (AP) and free carboxylic groups (OH) were selected for this study.

3.2. Pharmacological efficacy of doxorubicin bound to HPMA copolymer carrier through enzymatically non-cleavable GlyGly side chains; the effect of additional drug-free GlyPheLeuGly chains

The cytostatic activity of the conjugate containing doxorubicin bound to the HPMA copolymer through enzymatically non-cleavable GlyGly side chains (P-8) was compared with the conjugate containing additional drug-free sequences GlyPheLeuGly (P-9). Four cancer cell lines of mouse origin (T cell lymphoma EL4; B cell lymphoma 38C13; fibrobrosarcoma 3T3 and B cell leukemia BCL1) and three cancer cell lines of human origin (colorectal adenocarcinoma SW 620, B cell lymphoma Raji and T cell leukemia Jurkat) were tested using ^{3}H-thymidine incorporation to evaluate anti-proliferative and cytostatic capacity of the conjugates. Sample P-8 contained ≤0.1% of free doxorubicin and approx. 3 mol% of extra GlyGly side chains without the drug. Sample P-9 contained <0.1% of free doxorubicin, 4.6 mol% of extra GlyPheLeuGly side chains without the drug and approx. 3 mol% of GlyGly sequences without the drug. Data presented in Table 2 document that the cytostatic activity of a particular sample and a parent drug strictly depends on selected cancer cell line and that the variation in IC_{50} observed among different lines can reach as much as two orders of magnitude. Cytostatic activity of conjugate

Table 2
Comparison of cytostatic activity of conjugates P-8 and P-9

IC_{50} (μg/ml)							
Sample	EL4	38C13	3T3	BCL1	SW620	Raji	Jurkat
P-8	>40.0	11.17±5.9	4.7±1.5	0.47±0.11	17.1±7.4	0.31±0.21	>40.0
P-9	2.13±1.4	2.72±1.9	1.6±0.8	0.09±0.05	1.5±0.9	0.05±0.03	3.1±0.18
DOX	0.055±0.03	0.003±0.001	0.01±0.012	0.0003±0.0002	0.073±0.041	0.0002±0.0004	0.083±0.063

with doxorubicin bound through non-cleavable GlyGly sequence (P-8) is very low in EL4 and Jurkat lines (both originating from T cells) while the sensitivity of B cell lines (BCL1 and Raji) is higher by at least two orders of magnitude. In all seven cancer cell lines the low cytostatic efficacy of the conjugate P-8 can be considerably improved if the sample contains also extra drug-free GlyPheLeuGly side chains (conjugate P-9). The differences in IC_{50} are striking and varying from 2.9 times in cell lines relatively sensitive to the conjugate P-8 to 18.7 times in cell lines rather resistant to the conjugate P-8. Data are an average of six to eight experiments. Moreover, for the estimation of IC_{50} in each experiment we have used data from up to 50 individual wells containing radioactive DNA. The cytotoxicity-enhancing effect of extra GlyPheLeuGly side chains seems to be a general rule as it was repeatedly documented in all cancer cell lines we have used for the testing. The sensitivity of T lymphocytes of human (Jurkat) or mouse (EL4) origin to non-modified doxorubicin and to HPMA-based conjugates containing doxorubicin is low (Table 2). IC_{50} obtained with EL4 mouse T cell lymphoma and Jurkat human T leukemia is about 40 μg/ml. In such rather resistant systems the cytotoxicity-enhancing effect of tetrapeptide GlyPheLeuGly is quite obvious. The same holds for human colorectal adenocarcinoma SW 620 and mouse B cell lymphoma 38C13. The efficacy-enhancing effect of tetrapeptide GlyPheLeuGly is less striking but still seen in very drug-sensitive cancer cell lines, i.e. mouse B cell leukemia BCL1 and human B cell lymphoma Raji (Table 2).

3.3. Pharmacological efficacy of doxorubicin bound to HPMA copolymer carrier through enzymatically cleavable tetrapeptide GlyPheLeuGly by two different technologies; the effect of additional Gly, GlyGly, and GlyPheLeuGly side chains

The activity-influencing effect of drug-free oligopeptidic side-sequences was further studied by comparing of the activity of conjugates synthesized either by direct copolymerization of HPMA with monomeric doxorubicin (sample P-1) [20] or by polymer analogous reaction (sample PK1) [3,21]. Direct copolymerization, which starts from monomers terminated with the drug, results in a final product without drug-free GlyPheLeuGly sequences. On the other hand, after polymer analogous reaction drug-free sequences used as drug linkers always remain in the polymer structure. Table 3 shows quite clearly the superior activity of PK1 relative to P-1 in all six cancer cell lines tested. The activity-enhancing effect of GlyPheLeuGly tetrapeptide (sample PK1 versus P-1) was quite obvious in mouse B cell lymphoma 38C13, mouse fibrosarcoma 3T3, human B cell lymphoma Raji and human T cell leukemia Jurkat. The presence of extra drug-free sequences Gly (sample P-2) or GlyGly (sample P-3) did not considerably increase the anti-proliferative effect of HPMA copolymer-based conjugates.

Table 3
Comparison of cytostatic activity of conjugates PK1, P-1, P-2, P-3 and P-4

IC_{50} (μg/ml)						
Sample	EL4	38C13	3T3	SW620	Raji	Jurkat
PK1	32.3±7.7	2.01±0.74	2.8±0.78	12.38±5.85	0.22±0.09	17.55±8.4
P-1	>40.0	12.24±5.7	27.1±7.9	19.26±10.4	1.34±0.38	>40.0
P-2	>40.0	14.2±9.21	25.7±4.12	17.56±4.12	2.45±1.62	>40.0
P-3	>40.0	13.9±2.17	30.2±11.27	14.6±6.1	2.1±1.43	>40.0

Table 4
Comparison of cytostatic activity of conjugates PK1, P-4, P-5, P-6 and P-7

IC_{50} (μg/ml)						
Sample	EL4	38C13	3T3	SW620	Raji	Jurkat
PK 1	32.3±7.7	2.01±0.74	2.8±0.78	12.38±5.85	0.22±0.09	17.55±8.4
P-4	6.05±3.91	1.2±0.9	1.18±1.1	5.2±3.4	1.13±0.91	17.6±6.12
P-5	4.1±3.2	0.8±0.4	1.6±1.1	2.18±2.1	0.357±0.44	10.1±3.12
P-6	4.4±3.8	1.3±1.1	2.1±1.5	3.8±2.9	0.7±0.4	13.7±6.4
P-7	3.9±2.1	0.8±0.41	1.6±0.73	2.4±0.24	0.421±0.37	12.8±4.9

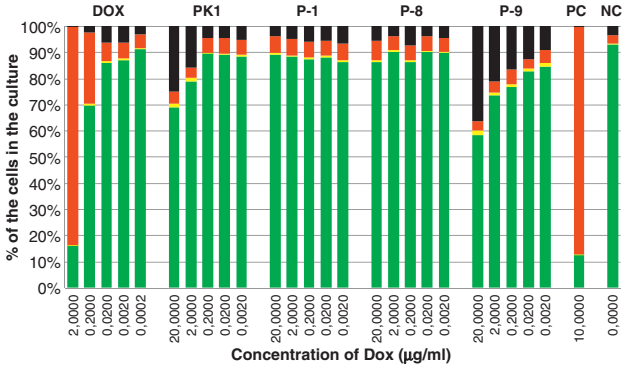

Fig. 1. Apoptosis and necrosis induced in EL4 thymoma cells. EL4 cells were incubated for 24 h in the presence of tested conjugates or free drug. Cells were stained with Annexin V-Dy643/Hoechst 33258 and the percentage of apoptotic cells was measured on flow cytometer BD LSR II. Cells incubated in fresh medium represent a negative control. As a positive control were taken cells incubated 12 h with doxorubicin (10 μg/ml). Live cells (AnnexinV negative/Hoechst 33258 negative). Dead cells (AnnexinV positive/Hoechst 33258 positive). Apoptotic cells (AnnexinV positive/Hoechst 33258 negative). Necrotic cells (AnnexinV negative/Hoechst 33258 positive).

3.4. Comparison of anti-proliferative effect of conjugates containing extra side chains GlyPheLeuGly terminated in AP, OH or NH_2

Conjugates P-4, P-5, P-6 and P-7 were synthesized by polymer analogous reaction and cytostatic activity was assessed using ^{3}H-thymidine incorporation. As we have repeatedly seen that additional oligopeptide side chains influence the pharmacological activity of the conjugate, we have compared conjugates similar to PK1 and containing extra GlyPheLeuGly side chains terminated either with hydrophilic 1-aminopropan-2-ol (P-4 and P-5), negatively charged COOH (P-6) or non-charged NH_2 group (P-7). The results presented in Table 4 show that conjugates with tetrapeptide GlyPheLeuGly ending with AP, NH_2 or COOH have a comparable anti-proliferative capacity. This means that the cytotoxicity of the conjugates is much more influenced by detailed amino acid composition of the pending oligopeptide (GlyPheLeuGly) than by its terminal group.

3.5. Apoptosis and necrosis induced in EL4 T cell lymphoma cells exposed to doxorubicin bound to HPMA-based copolymers differing in oligopeptide side chains

In EL4 T cell lymphoma cells, the presence of extra drug-free GlyPheLeuGly tetrapeptide linkers (sample P-9) in the polymeric conjugate considerably changes the ratio between necrotic and apoptotic cells. In the highest concentration used (20 μg/ml) the percentage of necrotic cells, i.e. cells positive for Hoechst 33258 and negative for Annexin V, was increased from less then 10% in cells exposed to the sample P-8 to almost 40% in cells exposed to the sample P-9 (Fig. 1, Table 5). Free doxorubicin induces mainly apoptosis (EL4 cells positive for Annexin V and negative for Hoechst 33258) while the percentage of apoptotic cells in all cultures exposed to HPMA conjugates (PK1, P-8, P-9) is very low (Fig. 1, Table 5). Contrary to apoptotic cells, whose percentage seems to be rather stable in the range of tested concentrations, the number of necrotic cells was concentration-dependent and was in full agreement with the cytostatic activity of the tested conjugate.

Table 5
Apoptosis and necrosis induced in EL4 mouse T cell lymphoma

Sample	μg/ml	Live	SD	Dead	SD	Apoptotic	SD	Necrotic	SD
PC	0	12.58%	6.2	0.06%	4.4	87.34%	3.2	0.02%	2.5
NC	10	92.84%	5.4	0.28%	6.7	3.40%	4.5	3.48%	1.6
DOX	2	16.37%	8.3	0.05%	11.2	83.54%	5.6	0.04%	0.6
DOX	0.2	69.75%	12.5	0.48%	7.3	27.37%	7.1	2.40%	1.5
DOX	0.02	85.74%	6.8	0.70%	9.8	7.07%	3.9	6.50%	2.4
DOX	0.002	87.10%	7.1	0.62%	4.3	5.97%	4.1	6.32%	2.2
DOX	0.0002	91.07%	9.3	0.49%	7.2	5.12%	2.2	3.32%	1.6
PK1	20	68.76%	7.8	1.48%	4.3	4.61%	3.2	25.15%	3.1
PK1	2	78.75%	8.3	1.38%	6.2	3.85%	2.9	16.02%	1.9
PK1	0.2	89.60%	9.0	0.24%	4.1	5.67%	4.5	4.49%	2.2
PK1	0.02	88.89%	12.4	0.46%	6.7	6.05%	6.9	4.60%	1.0
PK1	0.002	88.45%	4.5	0.44%	5.9	5.72%	4.8	5.38%	2.1
P-1	20	89.18%	13.1	0.44%	7.9	6.35%	7.1	4.03%	5.5
P-1	2	88.34%	9.5	0.47%	5.8	6.23%	5.4	4.96%	4.8
P-1	0.2	87.25%	12.4	0.56%	8.1	6.27%	7.1	5.91%	3.2
P-1	0.02	88.15%	10.0	0.53%	6.4	5.72%	3.8	5.61%	6.2
P-1	0.002	86.36%	9.1	0.59%	4.8	6.23%	4.4	6.82%	4.1
P-8	20	86.05%	8.5	0.94%	5.8	7.50%	4.5	5.51%	2.2
P-8	2	90.06%	6.3	0.58%	6.2	5.41%	2.8	3.95%	2.5
P-8	0.2	86.36%	12.4	0.67%	4.9	5.65%	5.1	7.33%	1.4
P-8	0.02	90.11%	9.7	0.42%	4.5	5.46%	6.3	4.01%	2.9
P-8	0.002	89.61%	7.6	0.32%	5.4	5.41%	4.7	4.66%	3.5
P-9	20	58.45%	5.8	1.61%	7.4	3.43%	4.2	36.51%	12.8
P-9	2	73.61%	10.9	0.94%	6.7	4.36%	5.1	21.09%	7.4
P-9	0.2	76.76%	9.1	1.01%	4.5	5.69%	3.6	16.54%	2.5
P-9	0.02	82.76%	7.3	0.82%	6.6	3.75%	4.2	12.66%	3.3
P-9	0.002	84.56%	8.5	1.43%	6.8	4.87%	6.1	9.15%	1.8

3.6. Intracellular accumulation of doxorubicin and HPMA-bound doxorubicin in normal Balb/c splenocytes and three cancer cell lines of mouse (T cell lymphoma EL4) and human (B cell lymphoma Raji and T cell leukemia Jurkat) origin

Cancer cells (1×10^6) and normal splenocytes (1×10^6) isolated from inbred Balb/c mice were incubated together in a mixed cell culture (ratio 1:1) with conjugates P-1, P-2, P-3, P-4, P-8, P-9 and PK1 at a concentration of 5 μg/ml for 24 h, washed with PBS and an intracellular accumulation of doxorubicin was measured as doxorubicin fluorescence on BD LSR II flow cytometer (Ex=488 nm, Em=580/25 nm). For easy identification, splenocytes were pre-labeled with DiD Vybrant labeling solution (Invitrogen, USA). Uptake by low-sensitive human T cell lymphoma Jurkat was compared with that of highly sensitive human B cell lymphoma Raji. Fig. 2 shows that accumulation of P-1, i.e. conjugate with no additional side chains, is lower compared to the conjugate PK1, i.e. conjugate containing additional GlyPheLeuGly side chains. Accumulation of conjugates with additional Gly (P-2) or GlyGly (P-3) side chains is comparable with the conjugate P-1. The highest accumulation was seen with a sample containing doxorubicin bound through non-cleavable sequence GlyGly and containing extra GlyPheLeuGly (P-9), which is comparable with the pharmacological activity detected as ^{3}H-thymidine uptake (see Fig. 2). The significant difference in uptake between samples P-8 and P-9 is quite obvious in all three cancer cell lines (mouse T cell lymphoma EL4, human B cell lymphoma Raji and human T cell leukemia Jurkat). An important finding is the fact that the accumulation of doxorubicin in the form of HPMA-based conjugates is always considerably lower in normal splenocytes if compared with cancer cells (Fig. 2). The highest uptake of all tested conjugates was observed with Raji cells, the lowest with Jurkat cells. In fact, the accumulation of the conjugates in selected cells and their cytotoxic effect are in a good agreement.

Cells exposed to conjugate P-9 reach maximal fluorescence intensity after 24 h and prolonged incubation for 48 h and 72 h does not increase the amount of accumulated doxorubicin

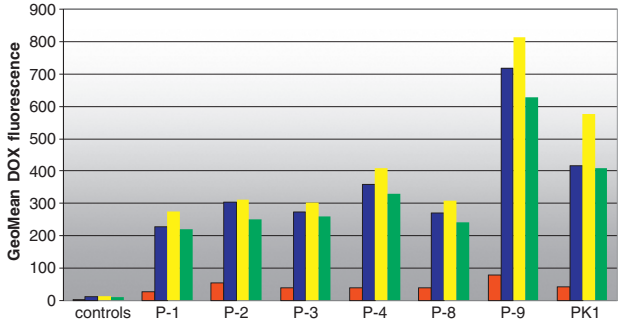

Fig. 2. Intracellular accumulation of different polymeric conjugates in selected cancer cell lines and normal Balb/c splenocytes. Mixture of normal splenocytes and selected cancer cell line was incubated with polymeric conjugates as described in Section 3.6. Splenocytes were pre-labeled with DiD Vybrant labeling solution (Invitrogen, USA). Conjugate accumulation was measured as doxorubicin fluorescence on BD LSR II flow cytometer (Ex 488, Em 580). Balb/c splenocytes. EL4 mouse T cell lymphoma. Raji human B-cell lymphoma. JURKAT human T cell lymphoma.

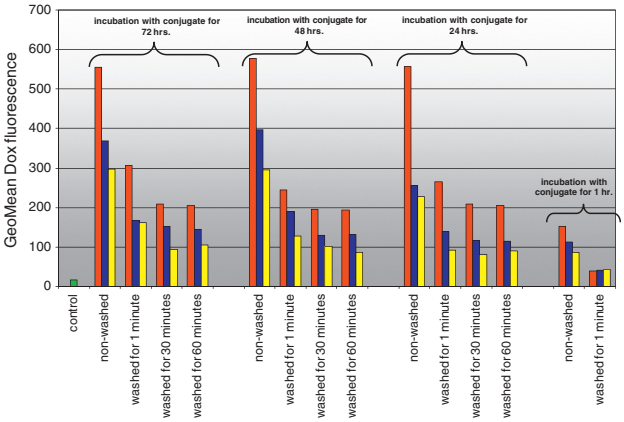

Fig. 3. The cellular uptake of different polymeric samples. EL4 T cell lymphoma cells were incubated with the conjugates PK1, P-8 and P-9 as described in the Section 3.6. untreated cells. cells treated with conjugate P-9. cells treated with conjugate PK1. cells treated with conjugate P-8.

(Fig. 3). On the contrary, PK1 and P-8 reach maximal fluorescence intensity after 48 h. However, their uptake is also fastest for the first 24 h when target cells accumulate about 80% of the final maximal fluorescence (Fig. 3). If the cells are washed with fresh medium, the intensity of fluorescence significantly decreases. Already one-minute washing causes a drop in the fluorescence intensity to 50% of that one detected in non-washed cells and with prolonged washing the fluorescence is decreased almost to 30% of initial values in all three tested conjugates (Fig. 3).

The explanation for a significant decrease in fluorescence after washing is complex and not that easy. It was published earlier [30] that PK1 accumulates in cells in seconds and that this accumulation is the same at 4 °C and 37 °C, which means that active transport is not involved and explanation is based on the physico-chemical interaction of the conjugate with the cell plasma membrane followed by intracellular accumulation. Most probably the interaction of a conjugate with cellular membranes is based on distribution coefficient in water/lipid phase. After washing, based on the same coefficient, substantial part of the conjugate moves back into the water phase. What is really interesting is the increase in the residual fluorescence suggesting that the conjugate moves during the first 24 h into other cellular compartments from which it is not released.

4. Discussion

A series of experiments performed in the eighties confirmed that lysosomal thiol-dependent proteases were crucial for degradation of oligopeptide spacers in HPMA-based polymer–drug conjugates [8] and GlyPheLeuGly sequence was selected as optimal for intracellular drug release and for the pharmacological activity of the HPMA conjugate [1,2]. The conjugates, including clinically tested PK1 and PK2, have been prepared by an aminolytic reaction of reactive polymer precursors with selected, mainly anticancer drugs. Unfortunately, a concomitant effect of the aminolytic reaction used in the synthesis of the conjugates consists in partial hydrolysis of ONp groups.

Moreover, after the drug is attached, the residual reactive ONp groups have to be eliminated from the polymer, usually by aminolysis with excess of 1-amino-2-propanol. This results in the formation of a drug conjugate with a small amount (approx. 3 mol%) of side chain oligopeptide sequences terminated in 2-hydroxypropyl amide and/or carboxylic groups. One of the goals of this work was to study the effect of such groups on the biological behavior of the conjugate. Pharmacological activity of the conjugate containing doxorubicin bound to the polymeric backbone through non-cleavable GlyGly side chains and an activity-enhancing ability of drug-free GlyPheLeuGly sequence was reported by us already in 2005 [22]. It was an unexpected and rather controversial observation. The first explanation was that the cytostatic activity of a non-cleavable conjugate is caused by traces of free drug. Thus, the conjugate was re-purified and *in vitro* experiments with cancer cells were repeated many times until it was quite certain that the pharmacological activity of conjugate with non-cleavable GlyGly side chains (P-8) and non-cleavable GlyGly side chains plus activity-enhancing drug-free GlyPheLeuGly chains (P-9) is not a consequence of contaminating parent drug. Control experiments with conjugates P-2 and P-3 revealed that the effect of additional GlyGly sequences (always present in GlyGly conjugates prepared by aminolysis) does not significantly influence the cytotoxicity of the conjugate and thus the cytotoxic effect of the conjugate with non-degradable spacer lies in a combination of a polymer and covalently bound drug.

Doxorubicin bound to HPMA copolymer via GlyGly spacer represents a conjugate where a drug is bound to the polymeric carrier through an enzymatically non-cleavable sequence while PK1 represents a conjugate where a drug is bound to the polymeric carrier through an enzymatically cleavable GlyPheLeuGly spacer. The relationship between the cleavability and pharmacological activity of the polymer therapeutics based on HPMA copolymer is still the subject of intensive and controversial discussion. Using our approach (visualization of doxorubicin by confocal microscope, BD LSR II, competition of the binding doxorubicin and/or Hoechst to nuclear DNA etc.) we are able to detect free doxorubicin in the nucleus after incubation of cells with a conjugate containing doxorubicin bound to the polymeric carrier through hydrazone, i.e. proteolytically cleavable bond [4,5]. However, using the same approach we are unable to determine free doxorubicin in the nucleus after exposure of the cells to PK1. We are not saying that doxorubicin cannot be intracellularly released from PK1 in some cells and might eventually appear in the nucleus. What we suggest is that it is probably not the main intracellular pathway of PK1.

According to our own experience the contamination with traces of doxorubicin can be a serious problem in the interpretation of obtained data. The paper published by M. Fiallo et al. [23] points out the fact that a new fluorescent spectrum is obtained after incubation of drug-sensitive cells with doxorubicin, which is due to 7,8-dehydro-9,10-desacetyldioxorubicinone formed in the medium. This compound, which is highly lipophilic, is taken up rapidly into cells and appears in the nucleus. Only highly purified samples without free doxorubicin and very short incubation could prevent misinterpretation of data obtained after long incubation with PK1 or after long manipulation with it. It cannot be excluded that instead of doxorubicin a highly fluorescent doxorubicin metabolite is determined in the nucleus.

Loadman et al. [24] report that the response of two murine colon tumor models correlates with cathepsin B activity as PK1 was more effective against MAC15A (tumor with high cathepsin B activity) compared to MAC26 (tumor with low cathepsin B activity). The current understanding of the mechanism of action of HPMA copolymer–anticancer conjugates is based on experiments *in vitro*, *in vivo* and also on clinical data [16,17,25]. However, the data interpretation is not an easy task as many factors act in concert to produce the observed cytostatic, cytotoxic and antitumor effect. Our recent observation of the action-enhancing effect of drug-free side tetrapeptide chains suggests that even more factors are in the play including the way of synthesis of the polymeric conjugate. One has to understand that at least the conjugates generally described as PK1, i.e. doxorubicin bound to HPMA copolymer carrier through GlyPheLeuGly spacer synthesized by direct copolymerization of HPMA with monomeric doxorubicin or by polymer analogous reaction are not the same. The precise level of free drug in tested conjugates as well as detailed description of structure of the carrier (MAG, HPMA copolymer of a PK1 or P-1 structure) has to be necessarily included in all papers to allow serious comparison of data obtained in different laboratories with apparently no quite identical samples.

Results given in Table 3 illustrate the advantages of use of the original method of synthesis of HPMA copolymers as carriers of anticancer drugs, i.e., aminolytic binding of doxorubicin onto polymer precursor containing GlyPheLeuGly–ONp side chains. Of course, the reproducibility of the reaction and determination of the exact structure are important aspects of the synthesis. The strategy of synthesis suggested by Caiolfa et al. [26] or others [27–29] starting from HPMA precursors bearing Gly–ONp reactive groups and their aminolysis with PheLeuGly derivatives of drugs (e.g., campthotecin or taxol) leads to polymeric products containing Gly–OH additional groups exhibiting lower anticancer activity than similar conjugates prepared by the classic method and containing additional GlyPheLeuGly groups in the conjugate structure (Table 3).

The present data as well as our previous results [19,30] do not support an idea of slow internalization of non-targeted PK1. We repeatedly demonstrated that intracellular localization of PK1 and P-1 is very fast and a considerable amount of doxorubicin can be detected intracellularly in seconds [30]. In fact, we pointed out that the efficacy of conjugates is also influenced by their rate of accumulation as early as 1989 [31]. The conjugate containing radioactive ^{125}I-daunomycin bound to HPMA copolymer carrier through GlyPheLeuGly sequence was accumulated in target cells more rapidly compared to the conjugate containing ^{125}I-daunomycin bound to the polymeric carrier through GlyGly linker. Duncan [10] suggests that in some cell types hydrophobic conjugates interact with the plasma membrane leading to a non-specific adsorptive uptake. We

have seen low accumulation in normal splenocytes, which seems to support an idea that the membrane morphology plays a role in non-specific uptake and intracellular accumulation of non-targeted polymeric drugs.

There are probably more factors responsible for increased activity of HPMA copolymer-based conjugates containing extra drug-free GlyPheLeuGly sequences. Only one of them might be a higher accumulation of such conjugates compared with those without such additional side chains. For reasons we do not yet completely understand the enhancing effect of the tetrapeptide was more obvious in a sample where doxorubicin was bound through more hydrophilic GlyGly dipeptide. As pharmacologically active conjugates such as PK1 synthesized by polymer analogous reaction always contained GlyPheLeuGly chains without the drug we suggest that this sequence in some way contributes to the efficacy of HPMA polymeric drugs, probably by influencing the cell surface binding, uptake and intracellular localization.

Our data clearly prove that also conjugates containing doxorubicin bound to the HPMA through non-cleavable sequence GlyGly inhibit proliferation of five out of seven tested cancer cell lines. Mouse B cell leukemia BCL1 and human B cell lymphoma Raji are the most sensitive. Such result supports the idea that the release of nucleotoxic doxorubicin from its polymeric carrier based on HPMA is not always a prerequisite for the pharmacological activity of such polymeric therapeutics and that other death-inducing mechanisms, such as membrane system collapse might be responsible for cell death [19,30]. It is necessary to stress that papers dealing with pharmacological efficacy of HPMA-based conjugates also documented a certain activity of conjugates containing a drug (daunorubicin or doxorubicin) bound through diglycine bond [1,12–15]. However, such an activity was always considerably lower than that observed with HPMA copolymer containing GlyPheLeuGly spacer. For example, we have shown *in vitro* and *in vivo* that anti-thymocyte antibody targeted conjugate containing daunomycin bound to HPMA copolymer carrier through GlyGly sequence was also active but its efficacy was considerably lower if compared with a similar conjugate where daunomycin was bound to the polymer backbone through GlyPheLeuGly side chain. Cytotoxicity *in vitro* was detectable mainly in the presence of complement [1,15]. Similarly, Duncan et al. [12] reported that *in vitro* inhibitory activity of non-targeted drug-conjugates against mouse leukemia L1210 and human lymphoblastoid leukemia CCRF is related to the amino acid composition of the drug–polymer linkage, conjugates with GlyPheLeuGly being more toxic compared to conjugates with GlyGly linker. P-GlyPheLeuGly–DOX conjugate with degradable spacer caused a significant increase in lifespan [32]. However, a comparison with similar conjugate containing GlyGly is not available as only GlyPheLeuGly containing carbohydrate-targeted conjugate was used. Our very recently obtained data using mouse T cell lymphoma EL4 confirm the original observation that conjugate containing doxorubicin bound to the polymeric carrier through GlyGly is only poorly effective *in vivo* [1,10,15,32]. We do not know why and we are working hard on the explanation. However, it is generally accepted that *in vitro* activity does not automatically mean *in vivo* activity as on the scene appear such factors as pharmacokinetics, EPR effect, transportation across endothelium, *in situ* accumulation of immunocompetent cells including T_{reg} cells and local activity of extracellular cancer cell-released proteases.

Presented data demonstrate that full understanding of the mechanism of action of polymer-based anticancer drugs requires careful definition of polymer carrier used and polymer conjugate structure and design of their new structures and methods of synthesis has to be selected with utmost care.

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References

[1] B. Říhová, J. Kopeček, Biological properties of targetable poly[*N*-(2-hydroxypropyl)methacrylamide]–antibody conjugates, J. Control. Release 2 (1985) 289–310.

[2] R. Duncan, H.C. Cable, J.B. Lloyd, P. Rejmanová, J. Kopeček, Polymers containing enzymatically degradable bonds. 7. Design of oligopeptide side chains in poly [*N*-(2-hydroxypropyl)methacrylamide] copolymers to promote efficient degradation by lysosomal enzymes, Makromol. Chem. 184 (1984) 1997–2008.

[3] J. Kopeček, P. Rejmanová, J. Strohalm, K. Ulbrich, B. Říhová, V. Chytrý, J.B. Lloyd, R. Duncan, Synthetic polymeric drugs (1991), U.S.Patent 5,037,883.

[4] T. Etrych, M. Jelínková, B. Říhová, K. Ulbrich, New HPMA copolymers containing doxorubicin bound via pH-sensitive linkage: synthesis and preliminary *in vitro* and *in vivo* biological properties, J. Control. Release 73 (2001) 89–102.

[5] T. Mrkvan, M. Šírová, T. Etrych, P. Chytil, J. Strohalm, D. Plocová, K. Ulbrich, B. Říhová, Chemotherapy based on HPMA copolymer conjugates with pH-controlled release of doxorubicin triggers anti-tumor immunity, J. Control.Release 110 (2005) 119–129.

[6] R. Duncan, J. Kopeček, J.B. Lloyd, Development of *N*-(2-hydroxypropyl) methacrylamide copolymers as carriers of therapeutic agents, in: E. Chiellini, P. Giusti (Eds.), Polymers in Medicine, Biomedical and Pharmacological Applications, Plenum Press, New York, 1983, pp. 97–113.

[7] J. Kopeček, P. Rejmanová, V. Chytrý, Polymers containing enzymatically degradable bonds. I. Chymotrypsin catalyzed hydrolysis of *p*-nitroanilides of phenylalanine and tyrosine attached to side-chains of copolymers of *N*-(2-hydroxypropyl)methacrylamide, Makromol. Chem. 182 (1981) 799–809.

[8] R. Duncan, H.C. Cable, J.B. Lloyd, P. Rejmanová, J. Kopeček, Degradation of side-chains of *N*-(2-hydroxypropyl)methacrylamide copolymers by lysosomal thiol–proteinases, Biosci. Rep. 2 (1982) 1041–1046.

[9] R. Duncan, P. Rejmanová, J. Kopeček, J.B. Lloyd, Pinocytic uptake and intracellular degradation of *N*-(2-hydroxypropyl)methacrylamide copolymers. A potential drug delivery system, Biochim. Biophys. Acta 678 (1981) 143–150.

[10] R. Duncan, *N*-(2-hydroxypropyl)methacrylamide copolymer conjugates, in: Glen S. Kwon (Ed.), Polymeric Drug Delivery Systems (Drugs and the Pharmaceutical Sciences), 148, 2007, pp. 1–92.

[11] P. Rejmanová, J. Kopeček, J. Pohl, M. Baudyš, V. Kostka, Polymers containing enzymatically degradable bonds,8[a]: degradation of oligopeptide sequences in *N*-(2-hydroxypropyl)methacryamide copolymers by bovine spleen cathepsin B, Makromol. Chem. 184 (1983) 2009–2020.

[12] R. Duncan, P. Kopečková-Rejmanová, J. Strohalm, I.C. Hume, H.C. Cable, J. Pohl, J.B. Lloyd, J. Kopeček, Anticancer agents coupled to *N*-(2-hydroxypropyl)methacrylamide copolymers. I. Evaluation of daunomycin and puromycin conjugates *in vitro*, Br. J. Cancer 55 (1987) 165–174.

[13] K.B. O'Hare, R. Duncan, J. Strohalm, K. Ulbrich, P. Kopečková, Polymeric drug-carriers containing doxorubicin and melanocyte stimulating hormone. *In vitro* and *in vivo* Evaluation Against Murine Melanoma, J. Drug Target 1 (1993) 217–229.

[14] L.W. Seymour, K. Ulbrich, S.R. Wedge, I.C. Hume, J. Strohalm, R. Duncan, *N*-(2-hydroxypropyl)methacrylamide copolymers targeted to the hepatocyte galactose-receptor: pharmacokinetics in DBA_2 mice, Br. J. Cancer 63 (1991) 859–866.

[15] B. Říhová, P. Kopečková, J. Strohalm, P. Rossmann, V. Větvička, J. Kopeček, Antibody-directed affinity therapy applied to the immune system: *in vivo* effectiveness and limited toxicity of daunomycin conjugated to HPMA copolymers and targeting antibody, Clin. Immunol. Immunopathol. 6 (1988) 100–114.

[16] P.A. Vasey, S.B. Kaye, R. Morrison, Ch. Twelves, P. Wilson, R. Duncan, A.H. Thomson, L.S. Murray, T.E. Hilditch, T. Murray, S. Burtles, D. Fraier, E. Frigerio, J. Cassidy, Phase I clinical and pharmacokinetic study of PK1 [*N*-(2-hydroxypropyl)methacrylamide copolymer doxorubicin]: first member of a new class of chemotherapeutic agents — drug–polymer conjugates, Clin. Cancer Res. 5 (1999) 83–94.

[17] P.J. Julyan, L.W. Seymour, D.R. Ferry, S. Daryani, Ch. M. Boivin, J. Doran, M. David, D. Anderson, Ch. Christodoulou, A.M. Young, S. Hesselwood, D.J. Kerr, Preliminary clinical study of the distribution of HPMA copolymers bearing doxorubicin and galactosamine, J. Control. Release 57 (1999) 281–290.

[18] M. Jelínková, J. Strohalm, D. Plocová, V. Šubr, M. Šťastný, K. Ulbrich, B. Říhová, Targeting of human and mouse T-lymphocytes by monoclonal antibody–HPMA copolymer–doxorubicin conjugates directed against different T-cell surface antigens, J. Control. Release 52 (1998) 253–270.

[19] O. Hovorka, M. Šťastný, T. Etrych, V. Šubr, J. Strohalm, K. Ulbrich, B. Říhová, Differences in the intracellular fate of free and polymer-bound doxorubicin, J. Control. Release 80 (2002) 101–117.

[20] N. Mongelli, E. Pesenti, A. Suarato, G. Biasoli, Polymer-bound paclitaxel derivatives. US Patent 1994: 5,362,831.

[21] J. Kopeček, Biodegradation of polymers for biomedical use, in: H. Benoit, P. Rempp (Eds.), IUPAC Macromolecules, Pergamon Press, Oxford, 1982, pp. 305–320.

[22] B. Říhová, V. Šubr, T. Etrych, P. Chytil, O. Hovorka, K. Ulbrich, Mistake lasting twenty years? To be *in vitro* effective doxorubicin does not have to be released from its polymeric HPMA-based carrier. Twelfth International Symposium on Recent Advances in Drug Delivery Systems, Salt Lake City, February 21–24 2005, p. 190, Abstracts.

[23] M. Fiallo, A. Laigle, M.N. Borrel, A. Garnier-Suillerot, Accumulation of degradation products of doxorubicin and pirarubicin formed in cell culture medium within sensitive and resistant cells, Biochem. Pharmacol. 45 (1993) 659–665.

[24] P.M. Loadman, M.C. Bibby, J.A. Double, W.M. Al-Shakhaa, R. Duncan, Pharmacokinetics of PK1 and doxorubicin in experimental colon tumour models with differing responses to PK1, Clin. Cancer Res 5 (1999) 3682–3688.

[25] B. Říhová, J. Strohalm, J. Prausová, K. Kubáčková, M. Jelínková, L. Rozprimová, M. Šírová, D. Plocová, T. Etrych, V. Šubr, T. Mrkvan, M. Kovář, K. Ulbrich, Cytostatic and immunomobilizing activities of polymer-bound drugs: experimental and first clinical data, J. Control. Release 91 (2003) 1–16.

[26] V.R. Caiolfa, M. Zamai, A. Fiorino, E. Frigerio, C. Pellizzoni, R. d'Argy, A. Ghiglieri, M.G. Castelli, M. Farao, E. Pesenti, M. Gigli, F. Angelucci, A. Suarato, Polymer-bound campthotecin: initial biodistribution and antitumour activity studies, J. Control. Release 65 (2000) 105–119.

[27] D. Fraier, E. Frigerio, G. Brianceschi, M. Casati, A. Benecchi, C. James, Determination of MAG-campthotecin, a new polymer-bound campthotecin derivative, and free campthotecin in dog plasma by HPLC with fluorimetric detection, J. Pharm. Biomed. Anal. 22 (2000) 505–514.

[28] N. Sarapa, M.R. Britto, W. Speed, M. Jannuzzo, M. Breda, C.A. James, M. Porro, M. Rocheti, A. Wanders, H. Mahteme, P. Nygren, Assessment of normal and tumour tissue uptake of MAG-CPT, a polymer-bound prodrug of campthotecin, in patients undergoing elective surgery for colorectal carcinoma, Cancer Chemother. Pharmacol. 52 (2003) 424–430.

[29] F.M. Wachters, H.J.M. Groen, J.G. Maring, J.A. Gietama, M. Porro, H. Dumez, G.E. de Vries, A.T. van Osterom, A phase I study with MAG-campthotecin intravenously administered weekly for 3 weeks in a 4-week cycle in adult patients with solid tumours, Br. J. Cancer 90 (2004) 2261–2267.

[30] O. Hovorka, T. Etrych, V. Šubr, J. Strohalm, K. Ulbrich, B. Říhová, HPMA based macromolecular therapeutics: internalization, intracellular pathway and cell death depend on the character of covalent bond between the drug and the peptidic spacer and also on spacer composition, J. Drug Target. 14 (2006) 391–403.

[31] B. Říhová, K. Vereš, L. Fornůsek, K. Ulbrich, J. Strohalm, V. Větvička, M. Bilej, J. Kopeček, Action of the polymeric prodrugs based on *N*-(2-hydroxypropyl)methacrylamide. II. Body distribution and T cell accumulation of free and polymer-bound [^{125}I] daunomycin, J. Control. Release 10 (1989) 37–49.

[32] R. Duncan, P. Kopečková, J. Strohalm, I.C. Hume, J.B. Lloyd, J. Kopeček, Anticancer agents coupled to *N*-(2-hydroxypropyl)methacrylamide copolymers. II. Evaluation of daunomycin conjugates *in vivo* against L1210 leukaemia, Br. J. Cancer 57 (1988) 147–156.

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New HPMA copolymer-based drug carriers with covalently bound hydrophobic substituents for solid tumour targeting

P. Chytil [a,*], T. Etrych [a], Č. Koňák [a], M. Šírová [b], T. Mrkvan [b], J. Bouček [b], B. Říhová [b], K. Ulbrich [a]

[a] Institute of Macromolecular Chemistry, Academy of Sciences of the Czech Republic, v. v. i., Heyrovský Sq. 2, 162 06 Prague 6, Czech Republic
[b] Institute of Microbiology, Academy of Sciences of the Czech Republic, v. v. i., Vídeňská 1083, 142 20 Prague 4, Czech Republic

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Abstract

Various conjugates of anticancer drug doxorubicin (Dox) covalently bound by the hydrolytically degradable hydrazone bond to the drug carrier based on *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymers were synthesised. Structure of the conjugates differed in the type and the content of hydrophobic substituent (dodecyl, oleic acid and cholesterol moieties) introduced into the polymer structure. In aqueous solutions the conjugates self-assembled into high-molecular-weight supramolecular structures, such as polymeric micelles or stable hydrophilic nanoparticles 13–37 nm in diameter, depending on the type and the content of hydrophobic substituents. Treatment of mice bearing EL-4 T cell lymphoma with the conjugates in the therapeutic regime of drug administration (i.v.) resulted in significant tumour regression with up to 100% of long-term survivors, depending on the dose and the detailed structure of the carrier. The nanoparticles formed by the conjugate bearing cholesterol moiety exhibited prolonged blood circulation and enhanced tumour accumulation indicating an important role of the EPR effect in excellent anticancer activity of the conjugate.

Keywords: HPMA copolymers; Drug carriers; Doxorubicin; Polymer micelles; EPR effect

1. Introduction

In recent years, development of new drugs and drug formulations based on water-soluble polymer drug carriers has been gaining importance. Most of these drug delivery systems have been intended for treatment of tumour diseases. Conjugation of drugs with polymers may reduce their toxicity, eliminate undesirable body interactions, improve their solubility, bioavailability and stability (enzymatic, thermal, etc.), and prolong blood clearance [1]. Moreover, polymer drug carriers may enable controlled drug release and specific delivery to the diseased or damaged tissue. Among the most intensively studied polymer drug carriers the copolymers based on *N*-(2-hydroxypropyl)methacrylamide (HPMA) are in the limelight [2,3]. Some of the polymer drug conjugates based on HPMA have been subjected to clinical trials [4].

Preferably, the drugs are covalently bound to the polymer carriers via spacers, which enable controlled release of active drug in the treated tissue or cells. Probably, most of the studied polymer drug carriers have been designed as lysosomotropic systems, where the drug could be released by lysosomal enzymes in lysosomes in tumour cells. In recent years pH-triggered drug release has been intensively studied. In this case the presence of enzymes is not essential, as the drug might be released in endosomes/lysosomes in tumour cells due to a decrease in pH from 7.4 (pH of blood) to 5–6 (pH of endosomes). For example, the hydrazone bond [5–8] or *cis*-aconityl linkage [8,9] have been employed as pH-sensitive linkages of drug to polymer carrier.

The high-molecular-weight of polymer carriers prevent fast release of the drug from the organism by renal filtration and, in this way, ensure prolonged blood circulation and retention of the drug in the body. Furthermore, macromolecules are more effectively accumulated in solid tumours due to the EPR (enhanced permeability and retention) effect [10]. The extent of

* Corresponding author. Tel.: +420 296 809 230; fax: +420 296 809 410.
E-mail address: chytil@imc.cas.cz (P. Chytil).

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passive accumulation of macromolecules in solid tumours strongly depends on their size and molecular weight. The EPR effect was observed for HPMA-based copolymers with molecular weights higher than 2×10^4 and grew with increasing molecular weight of polymers [11].

Probably most polymer drug carriers are synthetic polymers with non-biodegradable main chains. These polymers with molecular weight higher than the limit of renal filtration (ca. 5×10^4 for HPMA copolymers) cannot be eliminated from the body by kidneys filtration and hence undesired long-term accumulation of the carriers can occur in the body. Therefore, such high-molecular-weight polymer drug carriers have to contain biodegradable linkages within polymer chains in order to allow renal removal of the carrier from the body after fulfilling its function. Thus, various high-molecular-weight drug carriers consisting of branched copolymers based on HPMA [12,13], containing enzymatically degradable linkages, were prepared. Some of these conjugates showed significantly enhanced anticancer activity against selected tumours [6,14,15].

The size of carriers and molecular weight could also be increased using micellar structures formed by self-assembly of polymers with molecular weight lower than the limit of renal filtration. They are typically prepared by self-assembly of amphiphilic diblock copolymers into polymer micelle. The hydrophobic drug is incorporated into the hydrophobic core of the micelle physically (mostly by hydrophobic interactions) or by covalent bonds [16–19]. The hydrophobic core of the micelle is enveloped with a hydrophilic layer composed of a hydrophilic polymer, which protects the whole system from aggregation and undesired interactions with the components of living organisms.

Here, we describe the synthesis, physico-chemical and preliminary biological properties of new self-assembled drug delivery systems based on linear HPMA copolymers containing hydrophobic substituents, randomly distributed along the polymer chain. The basic structure of the drug conjugates consists of HPMA copolymer bearing anticancer drug doxorubicin (Dox) bound via the pH-sensitive hydrazone bond and a defined amount of dodecyl, oleic acid or cholesterol moieties differing in their hydrophobicity. Introduction of a proper amount of hydrophobic substituents into the polymer structure led to self-assembly of lower-molecular-weight polymers into high-molecular-weight supramolecular structures. Thus slower blood clearance, higher tumour accumulation and enhanced antitumour activity of the polymer drug can be expected due to the EPR effect.

2. Experimental part

2.1. Materials

1-Aminopropan-2-ol, methacryloyl chloride, 2,2′-azobis (isobutyronitrile) (AIBN), 6-aminohexanoic acid, methyl 6-aminohexanoate hydrochloride, hydrazine hydrate, *N*,*N*′-dicyclohexylcarbodiimide (DCC), *tert*-butyl carbazate, trifluoroacetic acid (TFA) and dimethyl sulfoxide (DMSO) were purchased from Fluka. Succinimidyl oleate was purchased from Sigma Aldrich. 2,4,6-Trinitrobenzene-1-sulfonic acid (TNBSA) was purchased from Serva, Heidelberg. Dodecyl methacrylate (DM) was purchased from Merck. Doxorubicin hydrochloride (Dox.HCl) was purchased from Meiji Seika. All reagents and solvents were of analytical grade. Solvents were dried and purified by conventional procedures and distilled before use.

2.2. Synthesis of monomers

N-(2-Hydroxypropyl)methacrylamide (HPMA), 6-methacrylamido hexanoic acid (MA-εAhx-OH), 1-(*tert*-butoxycarbonyl)-2-(6-methacrylamido hexanoyl)hydrazine (MA-εAhx-NHNH-Boc), 6-methacrylamido hexanoyl hydrazine (MA-εAhx-$NHNH_2$) were synthesised as described [20–23].

Cholest-5en-3β-yl 6-methacrylamido hexanoate (MA-εAhx-cholesterol) was prepared by the reaction of MA-εAhx-OH with cholesterol. 1.26 mmol of MA-εAhx-OH and 1.26 mmol of cholesterol were dissolved in 3.75 ml of fresh distilled tetrahydrofuran (THF). 1.51 mmol of DCC was dissolved in 0.75 ml of THF, a few crystals of 4-(dimethylamino)pyridine was added. Both solutions were cooled to −18 °C and mixed after 1-h cooling. The reaction mixture was left at −18 °C for 1 h, then at 4 °C for 16 h and at laboratory temperature for 1 h. Unreacted DCC was removed by reaction with 50 μl of acetic acid. After 30-min incubation, the precipitated *N*,*N*′-dicyclohexylurea was filtered off. The solution was concentrated to viscous oil and dissolved in ethyl acetate. Insoluble *N*,*N*′-dicyclohexylurea was filtered off again. Unreacted MA-εAhx-OH was extracted (five times) into an aqueous solution of $NaHCO_3$ (2 wt.%). Organic layers were dried with anhydrous Na_2SO_4 and the solvent was evaporated. The product was twice crystallized from acetone. Yield: 46%, m.p. 98–100 °C, elemental analysis: Calc. C 78.25%, H 10.83%, N 2.47%; Found C 78.73%, H 10.85%, N 2.34%, TLC: ethyl acetate/hexane (1:1), R_f=0.8. ^{1}H NMR ($CDCl_3$): δ 5.81 br, 1H (NH); δ 5.65 and 5.29 d, 2H (CH_2=C(CH_3)CO); δ 4.58 m, 1H (CO–O–C*H*–(CH_2)$_2$); δ 3.30 m, 1H (CH_2–NH); selected peaks of cholesterol part of molecule: δ 5.35 t, 1H (C=C*H*–CH_2); δ 0.66 s, 3H (C(18)H_3).

Purity of all monomers was examined by HPLC [Shimadzu HPLC system equipped with a reverse-phase column Tessek SGX C_{18} (125× 4 mm) and UV–VIS detector Shimadzu SPD-10AVvp (230 nm); eluent water–methanol with gradient 50–100 vol.% methanol, flow-rate 0.5 ml·min^{-1}].

2.3. Synthesis of polymer precursors

Polymer precursors **1a** and **1b** were synthesised by solution radical copolymerization of HPMA and MA-εAhx-NHNH-Boc in methanol using AIBN as initiator. AIBN (1 wt.%); monomers (14 wt.%); molar ratio HPMA/MA-εAhx-NHNH-Boc 92:8 **1a**, 89:11 **1b**. The copolymerization was followed by deprotection of Boc-protected hydrazide groups in TFA as described [22].

Polymer precursors **2a** and **2b** were prepared by solution radical terpolymerization of HPMA, MA-εAhx-NHNH-Boc and DM (distilled before use) in methanol using AIBN as

initiator. AIBN (1 wt.%); monomers (14 wt.%); molar ratio HPMA/MA-εAhx-NHNH-Boc/DM 90.5:8:1.5 **2a**, 87:8:5 **2b**. Reaction conditions, isolation of polymers and deprotection of hydrazide groups were carried out as described [22].

Polymer precursors **3a**, **3b** and **3c** were synthesised by reaction of succinimidyl oleate with a part of hydrazide groups of polymer precursors **1a** or **1b** [24]. The calculated theoretical contents of oleoyl groups were 1 mol% **3a**, 2 mol% **3b** and 5 mol% **3c**.

Polymer precursors **4a**, **4b** and **4c** were prepared by solution radical terpolymerization of HPMA, MA-εAhx-$NHNH_2$ and MA-εAhx-cholesterol in methanol using AIBN as initiator. AIBN (1 wt.%); monomers (14 wt.%); molar ratio HPMA/MA-εAhx-NHNH-Boc/MA-εAhx-cholesterol 90.5:8:1.5 **4a**, 89:8:3 **4b**, 87:8:5 **4c**. Reaction conditions and isolation of polymers were carried out as described [23].

2.4. Synthesis of polymer–Dox conjugates

Polymer–Dox conjugates **5**–**8** were prepared by the reaction of corresponding polymer precursors **1**–**4** containing hydrazide groups with Dox in methanol in the dark [25]. The polymer drug conjugates were purified from low-molecular-weight impurities (Dox or its degradation products) by gel filtration using a Sephadex LH-20 column and methanol as an eluent.

2.5. Physico-chemical characterisation

2.5.1. Determination of the content of functional groups in polymers

The content of hydrazide groups of polymer precursors was determined by the modified TNBSA assay. 25 μl of stock solution of copolymer containing hydrazide groups in DMSO (5 $mg \cdot ml^{-1}$) was added to a cuvette (l=1 cm, V=1 ml) containing 950 μl of the 9:1 mixture DMSO/borate buffer (0.1 M $Na_2B_4O_7.H_2O$, pH 9.3) and 25 μl of 0.03 M aqueous solution of TNBSA. After 45-min incubation, absorbance at λ=520 nm was measured. Molar absorption coefficient 21 130 $l \cdot mol^{-1} \cdot cm^{-1}$ used for calculation was estimated from a model reaction of MA-εAhx-$NHNH_2$ with TNBSA under the conditions described above. The content of residual hydrazide groups after drug attachment was estimated by subtraction of mol% of bound Dox in conjugates from mol% of hydrazides in respective polymer precursor.

The determination of dodecyl groups was performed by gas chromatography of dodecan-1-ol released by alkaline hydrolysis of the terpolymers using an external standard. 5 mg of each terpolymer was dissolved in 0.4 ml of 6 M NaOH and incubated at 110 °C for 13 h. The released dodecan-1-ol was extracted into 2 ml of ethyl acetate and this solution was analyzed by gas chromatography with a flame ionization detector (FID) [Gas chromatograph AutoSystem Perkin-Elmer, PE—Carbowax 20 M column (0.25 mm × 20 m, film thickness 0.25 μm), carrier gas nitrogen, detector temperature 240 °C, injector temperature 200 °C, oven temperature program: 100 °C for 2 min, then 8 °C/min up to 200 °C for 5.5 min].

The content of oleic acid derivate was determined by ^{1}H NMR (Bruker spectrometer, 300 MHz). Integral intensities from ^{1}H NMR spectrum in CD_3OD were compared: δ 5.35 t, 2H (CH═CH); δ 3.88 br, 1H (C*H*–OH).

The cholesterol content was determined by ^{1}H NMR. Integral intensities from ^{1}H NMR spectrum in $(CD_3)_2SO$ were compared: δ 5.32 t, 1H (C═CH); δ 4.70 br, 1H (C*H*–OH).

2.5.2. Determination of Dox content

The total content of Dox was measured by UV spectrophotometry in water [23]. The amount of unbound Dox was determined by SEC [Shimadzu HPLC system equipped with GPC column TSKgel G3000SWxl (300 × 7.8 mm; 5 μm) and UV–VIS detector Shimadzu SPD-10AVvp (488 nm); eluent methanol (80 vol.%) −0.3 M sodium acetate buffer (pH 6.5); flow-rate 0.5 $ml \cdot min^{-1}$] from the relative area of peak corresponding to the free drug and that corresponding to the sum of free and polymer-bound Dox.

The total Dox content in blood or tumour homogenates was performed by HPLC of 10-deoxyadriamycinon released by acid hydrolysis in 1 M HCl (50 °C; 1 h). 10-deoxyadriamycinon was extracted with chloroform, the organic phase was evaporated to dryness, dissolved in methanol and analyzed by HPLC [Shimadzu HPLC system equipped with a reverse-phase column Chromolith Performance RP-18e (100 × 4.6 mm; 5 μm) and fluorescence detector Shimadzu RF-10Axl (λ_{exc}=488 nm, λ_{em}=560 nm); eluent water–acetonitrile (0.1 vol.% TFA) with gradient 10–90 vol.% acetonitrile, flow-rate 1 $ml \cdot min^{-1}$]. The calibration was carried out by injection of the known amounts of free Dox to the blood and tumour homogenates obtained from untreated animals.

2.5.3. In vitro release of Dox from conjugates

The rates of Dox release were investigated in phosphate buffers at pH 5.0 or 7.4 (0.1 M, with 0.05 M NaCl) at 37 °C. The amount of released Dox was determined by SEC of the reaction mixture as described above. The values were a mean of three independent experiments.

2.5.4. Light scattering measurements

Molecular weight and polydispersity of polymers were determined by SEC [Shimadzu HPLC system equipped with GPC column TSKgel G3000SWxl (300 × 7.8 mm; 5 μm) and RI, UV–VIS and multiangle light scattering DAWN EOS (Wyatt Co.) detectors; eluent methanol–sodium acetate buffer (0.3 M; pH 6.5) (80: 20 vol.%); flow-rate 0.5 $ml \cdot min^{-1}$].

Hydrodynamic radii (R_H) and their distributions in physiological saline solution (0.15 M NaCl; polymer concentrations: 0.01 $g \cdot ml^{-1}$ for conjugates bearing hydrophobic substituents, 0.02 $g \cdot ml^{-1}$ for conjugates without hydrophobic substituents) were measured with a Nano-ZS instrument Zetasizer (ZEN3600, Malvern, UK). The intensity of scattered light was detected at angle θ=173°. The wavelength of laser was 632.8 nm. For evaluation of dynamic light scattering data, the DTS(Nano) program was used. The values were a mean of at least five independent measurements. Apparent molecular weight (M_{app}) of conjugates was analyzed by the Zimm procedure at angle θ=173°. Density of polymer coils, associates or micelles was determined using equation $\rho = M_{app}/(4/3 \cdot \pi \cdot (R_H)^3 \cdot N_A)$, where N_A is Avogadro constant. The aggregation number was determined as a ratio of M_{app} and M_w.

2.5.5. Determination of critical association concentration

The values of critical association concentration (CAC) of polymer precursors in water were estimated from the fluorescence intensity of the first peak (λ_{exc}=339 nm, λ_{em}=367 nm) of the pyrene fluoroprobe in solutions with different concentrations of the polymer [26].

2.6. Biological evaluation

2.6.1. In vitro cytostatic activity

Cytostatic activity of conjugates was assessed using a [^{3}H] thymidine incorporation assay. 96-well flat-bottomed microplates (NUNC, Denmark) were seeded with the following cancer cells: EL-4 mouse T cell lymphoma (5×10^4 cells/well); 38C13 mouse B cell lymphoma (2×10^3 cells/well); Raji human B cell lymphoma (1×10^4 cells/well); Jurkat human T cell leukemia (1×10^4 cells/well); BCL1 mouse B cell leukemia (5×10^3 cells/well); 3T3 mouse fibrosarcoma (1×10^4 cells/well); SW 620 human metastatic colorectal carcinoma (1×10^4 cells/well); FaDu human squamous-cell spinocellular carcinoma (5×10^4 cell/well). The tested samples (in triplicate) were then added to the wells to achieve the desired Dox concentrations (range 0.0002–40.0 μg·ml^{-1}). The plates were incubated in 5% CO_2 at 37 °C for 72 h (EL-4 cells for 48 h). 18.5 kBq (0.5 μCi) of [^{3}H]thymidine was added per well for the last 6 h (4 h for EL-4 cells, 5 h for 38C13 cells) of incubation. The cells were then collected on glass fibre filters (Filtermat, Wallac, Finland) using a cell harvester (Tomtec, U.S.A.) and the radioactivity of the samples was measured in a scintillation counter (1450 MicroBeta TriLux, Wallac, Finland). Cells cultivated in fresh medium were used as controls. The inhibition of tumour cell growth was expressed as IC_{50}, i.e., as the concentration of Dox (or Dox equivalent) inhibiting the cell growth by 50%. All IC_{50} values were a mean of at least three independent experiments.

2.6.2. In vivo antitumour activity

The antitumour efficacy of the conjugates was evaluated in C57BL/6 (B/6) mice with syngeneic T cell lymphoma EL-4 (ATCC TIB-39). The B/6 mice were from the Animal Facility of the Institute of Physiology, Academy of Sciences of the Czech Republic, v. v. i. The mice were injected subcutaneously (s.c.) into the shaven right ridge with 1×10^5 EL-4 cells to produce continuously growing solid tumours. Tumour sizes were measured with callipers in two perpendicular diameters every 3 days and expressed as tumour volume $V=a\times b^2/2$ (a— longer diameter, b — shorter diameter). The survival time was regularly scored. The conjugates were administered when the tumours were well established (5–8 mm in diameter, 8 or 9 days after transplantation), i.e., therapeutic regime of the treatment was employed. Conjugates **7c** and **8b** were injected i.v. either as a single dose (10 mg Dox(equivalent)/kg), or as two equal doses (total 10 mg Dox(equivalent)/kg) administered on days 8 and 12. Conjugate **5** was injected i.v. as a single dose (15 mg Dox (equivalent)/kg). The doses were determined according to mean body weight of the mice estimated on day 8. Groups of 8 mice were used for each administration. A retransplantation experiment was carried out in survived animals 115 days after the first tumour transplantation.

Mice were housed in accordance with approved guidelines. Food and water were given ad libitum. The animal room was maintained at 20 °C. The experimental designs were in accordance with the Act on Experimental Work with Animals (Decrees No. 311/97; 117/87, and Act No. 246/96) of the Czech Republic which is fully compatible with the corresponding European Union directives.

2.6.3. Blood clearance and tumour accumulation

The C57BL/6 (B/6) mice were subcutaneously implanted with 1×10^5 EL-4 cells. On day 8, conjugates **5** and **8b** (both 15 mg Dox(equivalent)/kg) and Dox.HCl (5 mg/kg) were

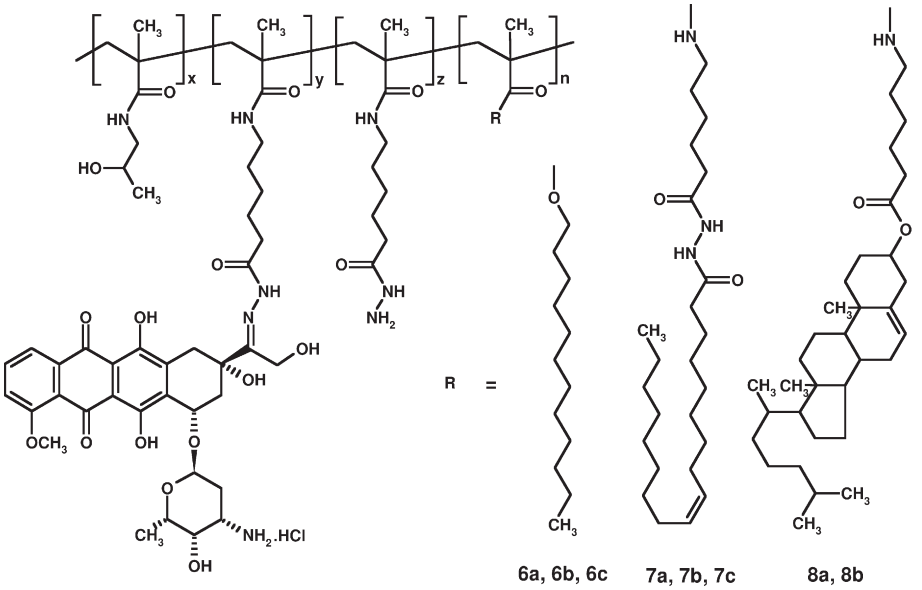

Fig. 1. Schematic structure of HPMA–doxorubicin conjugate bearing hydrophobic moieties.

Table 1
Physico-chemical characteristics of polymer precursors

Polymer precursor no.	Hydrophobic substituent		Content of $-NHNH_2$ (mol%)	M_w [a] ($\times 10^{-3}$)	M_w/M_n [a]	R_H [b] (nm)
	Type	Content (mol%)				
1a	–	–	5.3	24.2	1.7	3.8
1b	–	–	7.2	24.4	1.9	n.d.
2a	Dodecyl	1.6	5.1	22.9	2.1	6.7
2b	Dodecyl	4.7	4.3	29.5	2.4	5.6
3a [c]	Oleoyl	1.0	4.3	20.8	1.9	4.0
3b [c]	Oleoyl	1.9	3.4	19.2	1.9	6.8
3c [d]	Oleoyl	4.8	2.5	20.5	1.9	6.6
4a	Cholesteryl	1.4	5.5	16.3	2.2	8.8
4b	Cholesteryl	2.8	4.6	17.3	2.0	17.9
4c	Cholesteryl	5.0	4.4	20.5	2.3	n.d.

[a] Measured by GPC analysis (eluent: methanol/acetate buffer 80:20).
[b] Measured by QELS in 0.15 M NaCl.
[c] Prepared from polymer precursor **1a.**
[d] Prepared from polymer precursor **1b**.

administered i.v. as a single dose. The mice were randomized to groups of two per each time interval. Blood samples (from the tail vein) were taken from the first group of mice at 0.5, 1 and 3 h, and these mice were sacrificed 6 h after the conjugate administration. The other mice were sacrificed at 12, 24, 48, and 96 h after the drug injection. Again, blood samples were collected at each time interval, and the tumours were taken for analysis. The body weight of the mice was determined at each interval. The tissue samples were weighed, homogenized in a specified volume of phosphate buffered saline (PBS) using a glass homogenizer and the homogenates were stored at −20 °C before analysis. The samples were analyzed for the total Dox content, i.e., the sum of free and polymer-bound Dox, as described above.

3. Results and discussion

The aim of the study was to prepare efficient anticancer polymer drugs facilitating tumour-specific drug delivery. This paper focuses on the study of physico-chemical and biological behaviour of new supramolecular conjugates based on HPMA copolymers with pH-controlled drug release of Dox bound to the carrier by hydrazone bond. In our previous work, we showed that introduction of a highly hydrophobic substituent (oleic acid moiety) into the polymer structure led to self-assembly of polymer chains and to formation of high-molecular-weight polymer micelles [24]. In the present study, several conjugates differing in the content and chemical structure of hydrophobic substituents were investigated. Apart from oleoyl less hydrophobic dodecyl group and more hydrophobic cholesterol moiety were chosen.

The behaviour of these conjugates, in particular the impact of the substituents on the size and molecular weight of polymer coils, rates of *in vitro* drug release, biological properties such as *in vitro* cytostatic activity, *in vivo* anticancer activity and pharmacokinetics were studied. Detailed structures of the polymer conjugates are shown in Fig. 1. A list of physico-chemical characteristics of the polymer precursors is given in Table 1 and of the polymer–Dox conjugates in Table 2.

3.1. Synthesis of polymer precursors and polymer–Dox conjugates

Two methods of synthesis of polymer precursors containing hydrazide groups and hydrophobic substituents were used. The synthesis of polymer precursors **3a**, **3b** and **3c** started with water-soluble polymer precursors containing only hydrazide groups. (Conjugates of Dox prepared from this type of copolymers were studied and described in our previous papers [5,22,24]). Here, the hydrazide groups of polymer precursor **1a** or **1b** were partially modified by activated fatty acid prior to the reaction with Dox. The reaction with succinimidyl oleate was quantitative. Polymer precursors **2a**, **2b**, **4a**, **4b** and **4c** were prepared by terpolymerization of HPMA with comonomers bearing hydrazide groups and hydrophobic substituents. The commercially available dodecyl methacrylate and synthesised ester of cholesterol and 6-methacrylamido hexanoic acid were used. The yields of polymerizations varied between 65 and 75%; a significant influence of hydrophobic substituents on the yield was not observed. The composition of terpolymers approximately corresponded with the composition of the polymerization mixture thus suggesting that copolymerization parameters did not much differ from unity. All the polymer precursors (except **4c**) were used for conjugation with Dox. The reaction resulting in the hydrazone-bound Dox was not influenced by the presence of various additional groups introduced into the polymer structure. The yield of conversion of hydrazide groups was ~70%. The amount of residual

Table 2
Physico-chemical characteristics of polymer–Dox conjugates

Conjugate no.	Polymer precursor no.	Dox content (wt.%)	M_w [a] ($\times 10^{-3}$)	M_w/M_n [a]	R_H [b] (nm)	M_{app} [b] ($\times 10^{-3}$)	ρ [b] (g·cm^{-3})	n_a
5	**1a**	10.0	25.7	1.9	4.3	11	0.05	~1
6a	**2a**	7.7	27.2	1.9	6.1	22	0.04	~1
6b	**2b**	8.7	32.5	2.0	6.5	37	0.05	1.1
7a	**3a**	7.8	29.9	2.0	4.0	14	0.09	~1
7b	**3b**	7.8	32.4	2.1	6.4	110	0.17	3.4
7c	**3c**	6.1	29.0	1.9	6.4	170	0.26	5.9
8a	**4a**	8.6	23.7	2.0	9.6	41	0.02	1.7
8b	**4b**	8.7	24.5	2.2	18.4	460	0.03	19

[a] Measured by GPC analysis (eluent:methanol/acetate buffer 80:20).
[b] Measured by QELS in 0.15 M NaCl; $n_a = M_{app}/M_w$.

hydrazide groups in the conjugates after Dox attachment was similar in all the tested conjugates (around 2 mol%). Polymer precursors were of similar molecular weights as measured by SEC in a mixture of methanol/acetic buffer 80:20, which is assumed to disrupt any hydrophobic interactions. Their reaction with the drug did not lead to large changes of molecular weight.

3.2. Solution properties of conjugates

Accumulation of high-molecular-weight structures in solid tumours due to the EPR effect depends on their molecular weight (hydrodynamic size) [10,11]. Solution properties of both polymer precursors and conjugates were measured in physiological saline solution, which exhibited similar ionic strength as body fluids. Thus, the solution was used as an appropriate model of human body environment. Terpolymers were completely dissolved within 15–20 min using only stirring and ultrasound. The frequently used stepwise dialysis from a selective solvent to water [18,27] did not have to be applied.

An increase in hydrodynamic dimensions due to the self-assembly of conjugates bearing hydrophobic substituents was measured by quasi-elastic light scattering (QELS). Scattered light was evaluated from a back angle (173°), which was sufficient to determine hydrodynamic radii (R_H). However, molecular weights of conjugates calculated from the intensity of scattered light were only apparent molecular weights (M_{app}). Additionally, M_{app} were not extrapolated to zero concentration. Thus, M_{app} as well as ρ and n_a calculated using M_{app} might be interpreted only as relative values. The real molecular weight should be higher as demonstrate different values of M_{app} and M_w of conjugate **5**. This observation corresponds with the determined second virial coefficient A_2 for polymer–Dox conjugate containing similar amount of Dox [23]. The coefficient was positive ($A_2 = 5 \cdot 10^{-4}$), i.e., M_{app} increase with the decreasing concentration of copolymer.

R_H-distributions of conjugates represented by intensity of scattered light are shown in Fig. 2. R_H-distributions of polymer precursors (data not shown) were similar to corresponding polymer–Dox conjugates. The presence of hydrophobic substituents in the conjugate resulted in a significant change of its solution properties in aqueous solutions. Polymer micelles ($R_H \sim$ 6.5 nm) of a narrow size distribution were formed from conjugates **7b** and **7c** bearing the oleic acid moiety. M_{app} ($1–2 \times 10^5$) and ρ (~ 0.2 g·cm^{-3}) of the micelles increased approximately by one order of magnitude compared with linear conjugate **5**. Formation of micelles was confirmed by an increase in the aggregation number (4–6). The contents of hydrophobic group in conjugate **7a** and its polymer precursor **3a** were probably too low to enable formation of polymer micelles. The difference between M_{app} and M_w of conjugate **7a** might be explained in the same way as the difference in molecular weights of conjugate **5**.

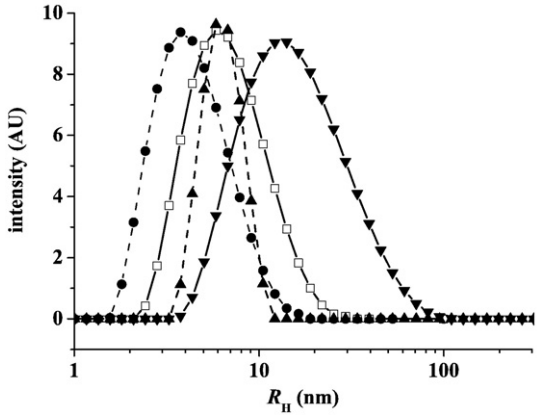

Fig. 2. Size distribution of conjugates in physiological solution; 5 (●- - -); 6b (□—); 7c (▲- - -); 8b (▼—).

Both **6a** and **6b** conjugates with dodecyl groups exhibited an increased size of polymer coils ($R_H \sim$ 6–6.5 nm) similarly as conjugates **7b** and **7c**. Nevertheless, their M_{app} did not grow rapidly even if a relatively high content of hydrophobic substituent was used. Also ρ or n_a did not increase and R_H-distribution was not narrow, i.e., typical characteristics of polymer micelles were not reached.

Conjugates **8a** and **8b** bearing cholesterol formed self-assembling supramolecular structures with the highest R_H (9–18 nm), even higher than R_H of conjugates **7b** and **7c**. Their M_{app} and n_a also increased significantly. In contrast to smaller micelles formed from conjugates containing the oleoyl groups, density (ρ) of micelles or nanoparticles formed by both conjugates bearing cholesterol did not grow with increasing R_H and also R_H-distribution was not very narrow. Their structures remind of high-molecular-weight random associates of flexible hydrophilic polymer chains connected by hydrophobic micelle core-like nodal points (point-like contacts) [28]. Due to the rather planar structure of cholesterol, it can be assumed that point-like contacts resemble sandwich-like structure of arranged cholesterol. Determination of the exact structure of associates will be the subject of our further interest. However, we assumed that nanoparticles of conjugate **8b** exhibiting the highest R_H might achieve a higher anticancer activity than other conjugates due to the EPR effect.

Critical association concentrations (CAC) were determined using pyrene fluoroprobe. Only polymer precursors containing the highest amount of hydrophobic groups (oleoyl and cholesterol) were tested. Unfortunately, we have not succeeded in testing corresponding Dox conjugates because the fluorescence spectrum of Dox overlaps that of pyrene. CAC for polymer precursor **3c** was 0.176 ± 0.009 g·l^{-1} and for **4b** 0.044 ± 0.002 g·l^{-1}. The CAC values were higher than those of PEG-based block copolymers studied by other authors, which typically vary from 0.001 to 0.010 g·l^{-1} [29]. The decrease in CAC was expected, because the length of hydrophobic substituents was much shorter than that of hydrophobic blocks of block copolymers. The shifted equilibrium between micelle and unimer can result in enhanced release of amphiphilic unimer from micellar carrier during blood circulation. The unimer could behave as a detergent and could disrupt cell membranes leading to the death of healthy cells. This is why *in vitro* experiments examining toxicity of the polymers were performed (See section "*In vitro* cytostatic activity").

3.3. In vitro drug release

In all conjugates Dox was bound to the polymer via pH-sensitive hydrazone bonds. Unlike micellar systems studied by

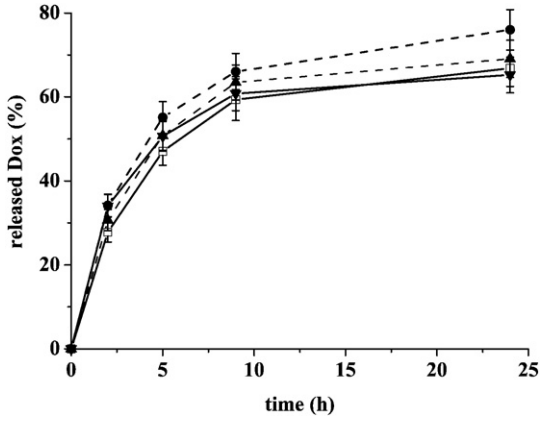

Fig. 3. Release of Dox from polymer–Dox conjugates incubated in phosphate buffer of pH 5.0 at 37 °C. 5 (●- - -); 6b (□—); 7c (▲- - -); 8b (▼—).

other authors where Dox was covalently attached or physically incorporated in polymer chains forming the hydrophobic core of micelles [17–19], in our case the drug was bound to a hydrophilic polymer forming the shell of micelles. Thus, Dox located in the shell, might be released more easily without overcoming the barrier of the core (steric hindrance and hydrophobic interactions).

Introduction of hydrophobic substituents into the polymer structure did not influence the rate of doxorubicin release at pH 7.4 at 37 °C and only ~5% Dox was released after 24 h incubation of all the studied conjugates; the detailed data are not shown. This result corresponds with the outcome of our previous study describing properties of soluble polymer–Dox conjugates bearing additional positively or negatively charged groups or hydrophobic substituents [24].

The rate of Dox release from the conjugate bearing the oleic acid moiety studied in the previous paper was determined using extraction from incubation buffer solutions into chloroform followed by HPLC analysis [24]. In this case, the rate of Dox release was significantly slower at pH 5 (~20% decrease after 24 h) than the rate of Dox release from linear water-soluble conjugate. Later on, we found that not all the released Dox was extracted into the organic layer and probably a part of the drug remained bound to the polymer via hydrophobic interactions in the solution. In this study, we applied a method which overcomes this disadvantage and uses direct analysis of incubation solutions by SEC using a mobile phase suppressing hydrophobic interactions [19].

The profiles of Dox release from selected conjugates incubated at pH modelling intracellular environment are given in Fig. 3. The highest rate of Dox release was found for conjugate **5** (76% Dox released after 24 h). All other tested conjugates showed only a slightly lower rate of Dox release (67% Dox released after 24 h from conjugate **6b**, 69% from **7c** and 65% from **8b**). To sum up, the presence of hydrophobic moieties in the polymer structure and physical structure did not significantly influence the rate of hydrolysis of hydrazone bonds at pH 5. The rate was by one order of magnitude higher than that at pH of blood.

3.4. In vitro cytostatic activity

The cytostatic activity of the conjugates was determined *in vitro* as their ability to inhibit cellular proliferation which was detected by [^{3}H]thymidine incorporation. Cancer cell lines of different origin and sensitivity to Dox.HCl were selected. All the tested conjugates exhibited quite high cytostatic activities (See Table 3). We found that the activity of the conjugates was strongly dependent on the cancer cell type. All the conjugates were highly toxic for cell lines sensitive to treatment with free doxorubicin (38C13 B cell lymphoma, BCL1 B cell leukemia, 3T3 fibrosarcoma, SW 620 human colorectal carcinoma, Raji human B cell lymphoma, FaDu human squamous-cell spinocellular carcinoma). In the above mentioned cell lines, IC_{50} values of conjugates **7c** and **8b** were in the same range as the IC_{50} value of conjugate **5** and no significant differences depending on the hydrophobic character of conjugates were observed. Even the tests with less Dox-sensitive mouse EL-4 T cell lymphoma and human Jurkat T cell leukemia did not show significant differences between cytostatic activities of the conjugates. Generally, the cytostatic activity of the conjugates was approximately by one order of magnitude lower than that of the free drug (IC_{50} of Dox.HCl was 0.010 μg Dox/ml for EL-4). As controls, polymer precursors containing only hydrophobic substituents **3c** and **4b** without drugs were also tested. They did not show any cytotoxic activity against the cancer cells even at very high concentrations, which were far beyond those used in *in vivo* experiments.

We are aware of the fact that the cytostatic activity of the conjugates can be influenced by the small amount of free Dox released to the incubation media. Thus, we consider IC_{50} values of the conjugates only as comparative values; we use them only for estimation of differences between various hydrazone samples, presuming the rate of their hydrolysis is almost the same.

Table 3
Cytostatic activity expressed as IC_{50} (μg Dox/ml)

	EL-4	38C13	Raji	Jurkat	BCL1	3T3	SW620	FaDu
3c	>400	141±63	>400	>400	118±36	129±53	>400	n.d.
4b	>400	255±97	>400	>400	240±98	367±127	>400	n.d.
5	0.275±0.090	0.011±0.008	0.006±0.003	0.595±0.250	0.040±0.030	0.040±0.010	0.035±0.010	0.017±0.020
7c	0.151±0.080	0.007±0.002	0.010±0.07	0.124±0.100	0.047±0.020	0.040±0.010	0.040±0.030	0.016±0.010
8b	0.159±0.070	0.005±0.001	0.013±0.010	0.120±0.040	0.032±0.030	0.040±0.020	0.040±0.040	0.023±0.020
Dox.HCl	0.010±0.008	0.001±0.001	0.001±0.002	0.119±0.090	0.001±0.001	0.006±0.002	0.003±0.004	0.005±0.001

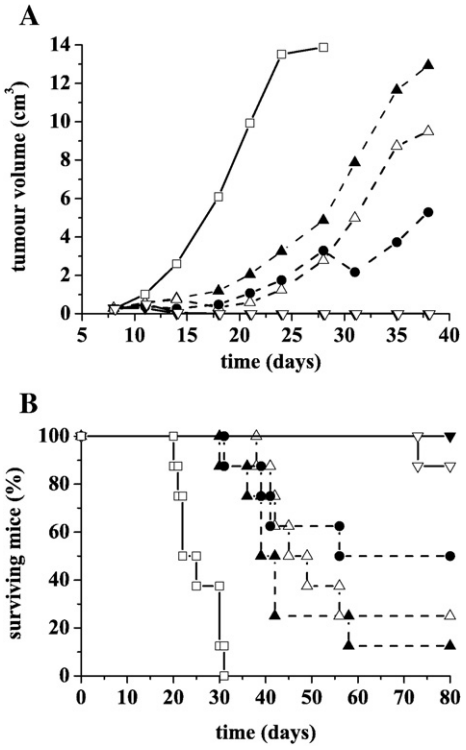

Fig. 4. *In vivo* effect of polymer–Dox conjugates on the growth of T cell lymphoma EL-4 (A), survival of mice (B); 5 (●- - -), 1×15 mg Dox(equivalent)/kg; 7c (▲- - -), 1×10 mg Dox(equivalent)/kg; 7c (Δ- - -), 2×5 mg Dox (equivalent)/kg; 8b (▼—), 1×10 mg Dox(equivalent)/kg; 8b (▽—), 2×5 mg Dox(equivalent)/kg; control (□—). All treated groups survived significantly longer compared with untreated control (Student's *T*-test; $P<0.05$).

3.5. In vivo anticancer activity

The anticancer activity of the oleoyl- and cholesterol-substituted conjugates was tested in the mouse syngeneic lymphoma model. The EL-4 tumours grow continuously following subcutaneous transplantation, and produce aggressive tumours which kill the untreated mice within 30–35 days (mean survival 32.5 time days, SD=1.034, median survival 32.6 days, $n=52$). No spontaneous regression was seen in the experiments. The substituted conjugates **7c** and **8b** were administered either as a single dose (1×10 mg Dox(equivalent)/kg) or as the same total dose, given in two parts (2×5 mg Dox(equivalent)/kg). The antitumour efficacy was compared with the efficacy of a suboptimal dose of unsubstituted conjugate **5** (15 mg Dox (equivalent)/kg), which was extensively studied earlier [5]. This suboptimal dose produced 50% of cured animals, in that the tumours regressed within 2 weeks following the treatment. The doses of both conjugates bearing hydrophobic substituents were lower than the dosage of conjugate **5**, because previously tested polymer–Dox conjugate containing the oleic acid moiety showed some signs of acute toxicity when administered as a single dose (1×15 mg Dox(equivalent)/kg) [24].

The oleoyl-substituted conjugate **7c** showed a weaker *in vivo* efficacy when compared with conjugate **5**, with no significant difference between the single dose and two partial doses (See Fig. 4). However, the conjugate extended survival of the treated mice significantly at each dosage ($P<0.01$). Probably, the size of micelles did not increase sufficiently in order to influence the EPR effect. The conjugate **8b** was highly effective against the EL-4 lymphoma, as it induced complete recovery of mice (100%) when injected as a single dose, and caused the tumour regression in 7 out of 8 mice (87.5%) when injected in two partial doses. In the last **8b**-treated animal, the tumour also regressed due to the treatment but resumed its growth after day 45 post transplantation. The survival time of that animal was highly extended when comparing with untreated controls (73 days). The higher antitumour efficacy of the single dose of the conjugate **8b** than the two doses seems to be in accordance with the assumption that the single dose allowed higher accumulation of the drug in the tumour tissue due to the EPR effect. The administration of conjugates **5** and **7c** was well tolerated, as no significant decrease in body weight or any discomfort were observed in the treated mice. Conjugate **8b** was quite well tolerated, but a slight temporary body weight loss less than 10% was detected in the animals at both dosages, peaking 7 days after the treatment, and recovering until day 30 post tumour transplantation.

It was already proven for other doxorubicin-containing HPMA-based copolymer conjugates that the treatment produced no serious damage to the immune system of the tumour-bearing mice, so that specific antitumour immunity developed in a significant proportion of the cured animals [5,30,31]. The mice with cured EL-4 lymphoma were retransplanted with the same tumour (1×10^5 EL-4 lymphoma cells s.c.) and left untreated. Despite the fact that the period between the first and second tumour transplantation was rather long (115 days), the three mice cured with the oleoyl-substituted conjugate **7c** were resistant, i.e., they did not develop any tumour after the retransplantation. A significant proportion of the **8b**-cured animals (8 mice cured with a single dose and 7 mice with two doses) also showed protection against the tumour growth (37% and 57%, respectively). The four mice cured with a suboptimal dose of conjugate **5** were also retransplanted with the challenge tumour, and three of them did not show any tumour growth after the retransplantation. Thus,

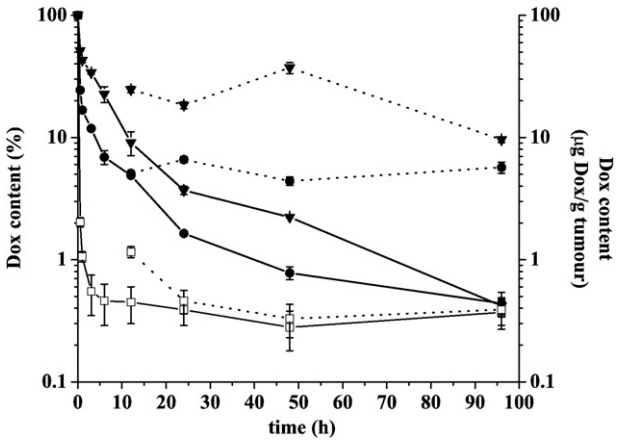

Fig. 5. Blood clearance and tumour accumulation of polymer–Dox conjugates and Dox.HCl; **5** (●), 1×15 mg Dox(equivalent)/kg; **8b** (▼), 1×15 mg Dox (equivalent)/kg; Dox.HCl (□), 1×5 mg Dox(equivalent)/kg; blood clearance (—, left *Y*-axis); tumour accumulation (·····, right *Y*-axis).

from these preliminary experiments it clearly results that the effective antitumour immune mechanisms could be activated during the treatment. The repeatedly observed fact that very efficient treatment stimulates lower antitumour reaction and vice versa was confirmed. The treatment with the hydrophobic substitution was not detrimental to the host immune system. Further experiments should be performed that would refine the dosages and treatment regime so that high efficacy in the primary tumour treatment and a significant protection against the tumour could be achieved. Speculatively, a moderate decrease in the dose of the cholesterol-substituted conjugate **8b** could retain the antitumour capacity of the treatment, but increase the proportion of the tumour-protected animals.

3.6. Blood clearance and tumour accumulation

Blood clearance of the cholesterol-substituted conjugate **8b** was evaluated and compared with that of the unsubstituted conjugate **5** and with the clearance of the free Dox.HCl. In accordance with increasing size of conjugates, the blood clearance of conjugate **8b** was much slower than the blood clearance of conjugate **5** (See Fig. 5). The Dox concentration in blood highly exceeded the concentration of conjugate **5** in the whole test interval (48 h). The conjugate **8b** was significantly accumulated in the tumour tissue. The Dox concentration in the tissue peaked 48 h after administration, reaching 35 μg Dox/g tumour, and dropping to 10 μg Dox/g detected at 96 h. Even after 96 h, the Dox content in the tumour was still significantly higher than that determined for conjugate **5**. However, the Dox accumulation following the administration of conjugate **5** was still very high in comparison with the free drug, in which the blood clearance was rapid and the accumulation in the tumour reached only negligible values after 12 h. Later on, the drug was hardly detectable in the tumour tissue as well as in blood. In total, the Dox accumulation of conjugate **8b** was approx. five times higher in comparison with conjugate **5** and sixty times higher compared with the free drug. The tumour-to-blood ratio of Dox (μg Dox/g tissue in tumour and blood) for both conjugates was increasing with time (from 0.6 after 12 h to 7.9 after 96 h for conjugate **5** and from 1.6 after 12 h to 14.0 after 96 h for conjugate **8b**), which indicated that the conjugates are passively accumulated in the tumour tissue due to the EPR effect. (The tumour-to-blood ratio of free Dox decreased from 2.4 after 12 h to 1.1 after 24 h and did not increase further).

4. Conclusion

HPMA-based copolymer–doxorubicin conjugates, differing in the type and the content of hydrophobic substituents (dodecyl, oleoyl and cholesterol), were synthesised. All the conjugates enabled pH-controlled activation of the drug in buffers modelling environment in endosomes/lysosomes of tumour cells (65–70% Dox released after 24 h, pH 5.0, 37 °C). The conjugates formed in aqueous solutions supramolecular structures, such as polymer micelles or stable associates, that were expected to show enhanced accumulation in solid tumours due to the EPR effect. In a preliminary biological evaluation, all the conjugates showed a high cytostatic activity for all tested tumour cell lines *in vitro*. Experiments in mice bearing mouse EL-4 T cell lymphoma showed slower blood clearance, enhanced tumour accumulation and significant antitumour activity with up to 100% of long-term survivors in the group of animals treated by the high-molecular-weight cholesterol-substituted conjugate. Hence, the activity of the conjugate was superior to that of the free drug and the shorter linear conjugate. We have also shown that the effective antitumour immune mechanisms could be activated during the treatment of tumour-bearing mice with HPMA copolymer–Dox conjugates.

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References

[1] R. Duncan, J. Kopeček, Soluble synthetic-polymers as potential-drug carriers, Adv. Polym. Sci. 57 (1984) 51–101.
[2] J. Kopeček, P. Kopečková, T. Minko, Z.R. Lu, HPMA copolymer–anticancer drug conjugates: design, activity, and mechanism of action, Eur. J. Pharm. Biopharm. 50 (2000) 61–81.
[3] R. Duncan, The dawning era of polymer therapeutics, Nat. Rev. Drug Discov. 2 (2003) 347–360.
[4] M.J. Vicent, R. Duncan, Polymer conjugates: nanosized medicines for treating cancer, Trends Biotechnol. 24 (2006) 39–47.
[5] T. Mrkvan, M. Šírová, T. Etrych, P. Chytil, J. Strohalm, D. Plocová, K. Ulbrich, B. Říhová, Chemotherapy based on HPMA copolymer conjugates with pH-controlled release of doxorubicin triggers anti-tumor immunity, J. Control. Release 110 (2005) 119–129.
[6] M. Pechar, A. Braunová, K. Ulbrich, M. Jelínková, B. Říhová, Poly (ethylene glycol)–doxorubicin conjugates with pH-controlled activation, J. Bioact. Compat. Polym. 20 (2005) 319–341.
[7] P.C.A. Rodrigues, T. Roth, H.H. Fiebig, C. Unger, R. Mulhaupt, F. Kratz, Correlation of the acid-sensitivity of polyethylene glycol daunorubicin conjugates with their *in vitro* antiproliferative activity, Bioorg. Med. Chem. 14 (2006) 4110–4117.
[8] K. Ulbrich, V. Šubr, Polymeric anticancer drugs with pH-controlled activation, Adv. Drug Deliv. Rev. 56 (2004) 1023–1050.
[9] W.M. Choi, P. Kopečková, T. Minko, J. Kopeček, Synthesis of HPMA copolymer containing adriamycin bound via an acid-labile spacer and its activity toward human ovarian carcinoma cells, J. Bioact. Compat. Polym. 14 (1999) 447–456.
[10] H. Maeda, Y. Matsumura, Tumoritropic and lymphotropic principles of macromolecular drugs, Crit. Rev. Ther. Drug Carr. Syst. 6 (1989) 193–210.
[11] L.W. Seymour, Y. Miyamoto, H. Maeda, M. Brereton, J. Strohalm, K. Ulbrich, R. Duncan, Influence of molecular-weight on passive tumor accumulation of a soluble macromolecular drug carrier, Eur. J. Cancer 31A (1995) 766–770.
[12] M. Dvořák, P. Kopečková, J. Kopeček, High-molecular weight HPMA copolymer–adriamycin conjugates, J. Control. Release 60 (1999) 321–332.
[13] T. Etrych, M. Jelínková, B. Říhová, K. Ulbrich, New HPMA copolymers containing doxorubicin bound via pH-sensitive linkage: synthesis and preliminary *in vitro* and *in vivo* biological properties, J. Control. Release 73 (2001) 89–102.
[14] J.G. Shiah, M. Dvořák, P. Kopečková, Y. Sun, C.M. Peterson, J. Kopeček, Biodistribution and antitumour efficacy of long-circulating *N*-(2-hydroxypropyl)methacrylamide copolymer–doxorubicin conjugates in nude mice, Eur. J. Cancer 37 (2001) 131–139.
[15] K. Ulbrich, T. Etrych, P. Chytil, M. Jelínková, B. Říhová, HPMA copolymers with pH-controlled release of doxorubicin—*in vitro* cytotoxicity and *in vivo* antitumor activity, J. Control. Release 87 (2003) 33–47.

[16] K. Kataoka, A. Harada, Y. Nagasaki, Block copolymer micelles for drug delivery: design, characterization and biological significance, Adv. Drug Deliv. Rev. 47 (2001) 113–131.
[17] H.S. Yoo, E.A. Lee, T.G. Park, Doxorubicin-conjugated biodegradable polymeric micelles having acid-cleavable linkages, J. Control. Release 82 (2002) 17–27.
[18] Y. Bae, N. Nishiyama, S. Fukushima, H. Koyama, M. Yasuhiro, K. Kataoka, Preparation and biological characterization of polymeric micelle drug carriers with intracellular pH-triggered drug release property: tumor permeability, controlled subcellular drug distribution, and enhanced *in vivo* antitumor efficacy, Bioconjug. Chem. 16 (2005) 122–130.
[19] M. Hrubý, Č. Koňák, K. Ulbrich, Polymeric micellar pH-sensitive drug delivery system for doxorubicin, J. Control. Release 103 (2005) 137–148.
[20] K. Ulbrich, V. Šubr, J. Strohalm, D. Plocová, M. Jelínková, B. Říhová, Polymeric drugs based on conjugates of synthetic and natural macromolecules I. Synthesis and physico-chemical characterisation, J. Control. Release 64 (2000) 63–79.
[21] J. Drobník, J. Kopeček, J. Labský, P. Rejmanová, J. Exner, V. Saudek, J. Kálal, Enzymatic cleavage of side chains of synthetic water-soluble polymers, Makromol. Chem. 177 (1976) 2833–2848.
[22] K. Ulbrich, T. Etrych, P. Chytil, M. Jelínková, B. Říhová, Antibody-targeted polymer–doxorubicin conjugates with pH-controlled activation, J. Drug Target. 12 (2004) 477–489.
[23] T. Etrych, T. Mrkvan, P. Chytil, Č. Koňák, B. Říhová, K. Ulbrich, New methods of synthesis of polymer prodrugs based on HPMA conjugates with pH-controlled activation of doxorubicin, J. Appl. Polym. Sci. (submitted for publication).
[24] P. Chytil, T. Etrych, Č. Koňák, M. Šírová, T. Mrkvan, B. Říhová, K. Ulbrich, Properties of HPMA copolymer–doxorubicin conjugates with pH-controlled activation: effect of polymer chain modification, J. Control. Release 115 (2006) 26–36.
[25] T. Etrych, P. Chytil, M. Jelínková, B. Říhová, K. Ulbrich, Synthesis of HPMA copolymers containing doxorubicin bound via a hydrazone linkage. Effect of spacer on drug release and *in vitro* cytotoxicity, Macromol. Biosci. 2 (2002) 43–52.
[26] E.S. Lee, H.J. Shin, K. Na, Y.H. Bae, Poly(L-histidine)-PEG block copolymer micelles and pH-induced destabilization, J. Control. Release 90 (2003) 363–374.
[27] Č. Koňák, B. Ganchev, M. Teodorescu, K. Matyjaszewski, P. Kopečková, J. Kopeček, Poly [*N*-(2-hydroxypropyl)methacrylamide-block *n*-butyl acrylate] micelles in water/DMF mixed solvents, Polymer 43 (2002) 3735–3741.
[28] W. Burchard, Macromolecular association phenomena: a neglected field of research, Trends Polym. Sci. 1 (1993) 192–198.
[29] M. Yokoyama, in: G.S. Kwon (Ed.), Polymeric Drug Delivery Systems, Taylor & Francis, 2005, pp. 533–575.
[30] B. Říhová, J. Strohalm, M. Kovář, T. Mrkvan, V. Šubr, O. Hovorka, M. Šírová, L. Rozprimová, K. Kubáčková, K. Ulbrich, Induction of systemic antitumour resistance with targeted polymers, Scand. J. Immunol. 62 (2005) 100–105.
[31] M. Šírová, J. Strohalm, V. Šubr, D. Plocová, P. Rossmann, T. Mrkvan, K. Ulbrich, B. Říhová, Treatment with HPMA copolymer-based doxorubicin conjugate containing human immunoglobulin induces long-lasting systemic anti-tumour immunity in mice, Cancer Immunol. Immunother. 56 (2007) 35–47.

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Nucleostemin Expression in Squamous Cell Carcinoma of the Head and Neck

ZDENEK CADA[1,2], JAN BOUCEK[2,3], BARBORA DVORANOVA[1,4], MARTIN CHOVANEC[1,2,4], JAN PLZAK[1,2,4], ROMAN KODET[5], JAN BETKA[2], GIAN L. PINOT[1], HANS-JOACHIM GABIUS[6] and KAREL SMETANA Jr.[1,4]

[1]Institute of Anatomy and [2]Department of Otorhinolaryngology, Head and Neck Surgery, First Faculty of Medicine and [4]Center of Cell Therapy and Tissue Repair and [5]Institute of Pathology and Molecular Medicine, Second Faculty of Medicine, [3]Institute of Microbiology, Academy of Science of the Czech Republic, Prague, Czech Republic; [6]Institute of Physiological Chemistry Ludwig-Maximilians-University, Faculty of Veterinary Medicine, Munich, Germany

Abstract. *Background: This study presents initial data on presence of nucleostemin – a nucleolar protein typical for stem cells in the normal squamous epithelium of the oropharynx and larynx – in squamous cell carcinoma originating from these epithelia. Materials and Methods: Differentiation and proliferation markers such as keratins, β-catenin, galectin-1, and Ki67 were studied in parallel with nucleostemin for defining cell characteristics. Results: Nucleostemin was detected in nucleoli of both proliferating basal cells and terminally differentiated suprabasal cells of normal epithelium and in tumor cells. Importantly, malignant transformation was connected with a significant enlargement of nucleostemin-positive nucleoli in these cell types. Conclusion: Therefore, the pattern of nucleostemin presence deserves as new marker for evaluation of tumor differentiation and biology.*

Head and neck cancers represent about 6% of malignant tumor cases worldwide; at least 90% of these tumors are squamous cell carcinomas. Despite rapid progress in diagnosis and therapy the overall 5-year survival rate for this malignancy is among the lowest of the major cancer types (1). This unfavorable situation calls for research activities to aim at finding new markers to better characterize the biological behavior of tumors in order to serve as a rational guideline to improve therapeutic modalities (2-5). Respective candidates may originate from applying the stem cell concept to this tumor class.

Adult tissue stem cells have several similarities with cancer cells, and the idea of stem cells as a source of solid cancer was put forward recently (6, 7). As a consequence, potential roles of epidermal stem cells in cancer, especially in squamous cell carcinoma, have been proposed (8). Fitting this concept, characteristics of the epidermal stem cell phenotype could be detected in *in vitro* propagated cells from cancer lines of squamous cell epithelial origin (9, 10). Moreover, cells of a very low differentiation level, akin to epidermal stem cells, have been observed on the periphery of tumor lesions in the so-called "aggressive front" of carcinomas. Tumors abundantly populated by these cells exhibit a highly anaplastic aggressive phenotype (11). At present, no single specific marker of adult tissue stem cells (including stem cells of squamous epithelia) has yet been discovered. These cells are currently identified by the detection of a combination of markers. In this situation, the systematic study of individual proteins will help characterize the phenotype of these cells thoroughly. This rationale prompts the study of nucleostemin, a nuclear/nucleolar protein present in neural and bone marrow stem cells and their related malignancies (12, 13). Nucleostemin, of note, participates in the control of proliferation in these cells and also in early embryonic development (14) and tissue regeneration (15), explaining why monitoring of its presence in cancer is warranted. In the human epidermis, this protein is not exclusively expressed by cells of the stem cell pool, and even nucleoli of terminally differentiated suprabasal cells reveal the presence of nucleostemin (16). However, nucleostemin expression is up-regulated in follicular bulge epidermal stem cells when measured by microarray technology at the mRNA level (17); *in vitro* only those cells cocultured with non-tumor feeder cells contain nucleoli positive for nucleostemin expression (16).

This study demonstrates the expression of nucleostemin in nucleoli of cells of normal squamous cell epithelium (namely of the larynx and oropharynx) and in squamous cell carcinomas originating from these epithelia. The scope of these results was extended to nucleostemin presence in FaDu cells, a model line of human squamous cell carcinoma

Correspondence to: Karel Smetana Jr., Charles University, First Faculty of Medicine, Institute of Anatomy, U nemocnice 3, 128 00 Prague 2, Czech Republic. Tel: +420 2 24965873, Fax: +420 2 24965770, e-mail: karel.smetana@lf1.cuni.cz

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from the hypopharynx, *in vitro* and *in vivo* after tumor development in nu/nu mice. To relate nucleostemin presence to other cellular characteristics, the presence of the following well-established markers was determined: Ki-67, β-catenin, and keratin 10. Proliferating cells were detected by the nuclear expression of Ki67 (18). β-Catenin is usually a membrane-associated protein in the majority of cells of the squamous cell epithelium; its shift to the cytoplasm and nucleus is related to with tumor progression (19). Keratin 10 expression is associated with terminal differentiation in cells of squamous epithelia under physiological conditions and in cancer (11, 20). In addition, the presence of a key member of the adhesion/growth-regulatory galectins, galectin-1 was determined. These endogenous lectins can interact with distinct glycan epitopes and proteins at different sites of the cell to trigger efficient signaling leading to diverse cell responses (21-23). In this context it is noteworthy that nuclear presence of galectin-1 has been observed in cells of the bulge region of the hair follicle which are phenotypically similar to epidermal stem cells (24).

Materials and Methods

Clinical material. Five specimens of laryngeal squamous cell carcinoma, three specimens of squamous cell carcinoma of the tongue and four specimens of oropharyngeal squamous cell carcinoma at stage T3 and without previous therapy as well as five control samples of normal laryngeal mucosa and three control samples of oropharyngeal mucosa (control samples were obtained from tumor-free organs as verified by histology) were taken. All samples were donated with the informed consent of the patients. The tissue donors had not undergone previous cytostatic (chemo)therapy. The samples were frozen in liquid nitrogen using Tissue-Tek (Christine Gröpl, Tulln, Austria) as a cryoprotective medium and stored at –85˚C until further processing.

Tissue culture and animal experiments. The human hypopharyngeal squamous cell carcinoma line FaDu (HTB-43) was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA), and the cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum, antibiotics (100 units/mL of penicillin, 100 μg/mL of streptomycin; Sigma, St. Louis, MO, USA), 1.5 g/L $NaHCO_3$, 0.11 g/L sodium pyruvate, 0.292 g/L *L*-glutamine, and 10 mM *N*-(2-hydroxyethyl) piperazine-*N*'-(2-ethanesulfonic acid) (HEPES). FaDu cells were also cultured on coverslips as described previously (10, 16). The cells were cultured under standard conditions *i.e.* under 5% CO_2 tension at 37˚C. Three independent experimental series were immunohisto-chemically evaluated. For xenotransplantation, two female nu/nu CD-1 mice, aged 8-12 weeks, were purchased from the Institute of Molecular Genetics, Academy of Sciences of the Czech Republic. The mice were housed in accordance with approved guidelines and provided food and water *ad libitum*. A total of $1x10^6$ FaDu cells from tissue culture were resuspended in 100 μl of phosphate buffered saline (PBS) and mixed with 50 μl of BD Matrigel™ (BD Biosciences, Erembodegen, Belgium) according to supplier instruction. The resulted suspension was then subcutaneously injected into each nu/nu CD-1 female mice.

The animals were sacrificed after 49 days and the specimens were frozen as described above.

Immunohistochemistry. Frozen sections, 7 μm each, were prepared using Cryocut E (Reichert-Jung, Vienna, Austria). The tumor sections and the FaDu cells grown on coverslips were washed with PBS, briefly fixed with 4% paraformaldehyde in PBS (pH 7.3) at room temperature, and then washed once with PBS. Diluted porcine serum (1%) (DAKO, Brno, Czech Republic) was used as a blocking solution to prevent the nonspecific binding of first and second step antibodies. Nucleostemin was detected by goat polyclonal antibody (Neuromics, Bloomington, MN, USA). Ki-67, a pankeratin, and keratin 10 were visualized by commercial mouse monoclonal antibodies (DAKO, Brno, Czech Republic) and β-catenin by a rabbit polyclonal antibody (Santa Cruz, Santa Cruz, CA, USA). Rabbit polyclonal antibody against galectin-1 (non-crossreactive with other galectins) was employed to visualize this antigen (25, 26). TRITC-labeled donkey anti-goat (Jackson Laboratories, West Grove, PA, USA) along with FITC-labeled swine anti-mouse (SwAM-FITC, AlSeVa, Prague, Czech Republic) and FITC-labeled swine anti-rabbit (SwAR-FITC, AlSeVa, Prague, Czech Republic) were used as second-step reagents. All commercial antibodies were diluted according to supplier recommendations. Five sections from the each tumor samples were employed for the each antibody combinations. Sections and cultured cells were stained at room temperature for 60 minutes. Specificity controls were performed by omitting the first-step antibody or by replacing it with monoclonal/polyclonal antibodies against thyroglobulin (not expressed in the studied tissues; DAKO, Brno, Czech Republic) to exclude any interaction of an antibody with sections of the studied tissues *via* Fc receptor. The nuclei were then counterstained with DAPI (4',6'-diamidino-2-phenylindole dilactate) (Sigma-Aldrich, Prague, Czech Republic). The specimens were mounted using Vectashield (Vector Laboratories, Burlingame, CA, USA) to prevent the UV bleaching of fluorochromes. A Nikon Eclipse-90i fluorescence microscope (Nikon, Prague, Czech Republic) equipped with filter blocks specific for DAPI, FITC and TRITC, a cooled CCD Vosskühler Cool-1300Q camera (Vosskühler, Osnabrick, Germany) and a computer-assisted image analyzer LUCIA 5.1 (Laboratory Imaging, Prague, Czech Republic) were used for imaging. The image analyzer was also used for measuring the size of nucleolar area positive for nucleostemin. A total of 300-500 cells were analyzed in each specimen. The results were statistically processed using Student's unpaired *t*-test.

Results

Normal oropharyngeal and laryngeal epithelium exhibited nucleostemin-positive nucleoli in both basal and suprabasal cells (Figure 1A). This observation is in accordance with a previous study of normal epidermis, hereby serving as internal quality control (16). The size of nucleostemin-positive nucleoli was identical in both compartments (Figure 2A). In order to support this notion the presence of Ki-67was measured and found to be restricted to cells of the basal layer in samples of normal epithelium (not shown).

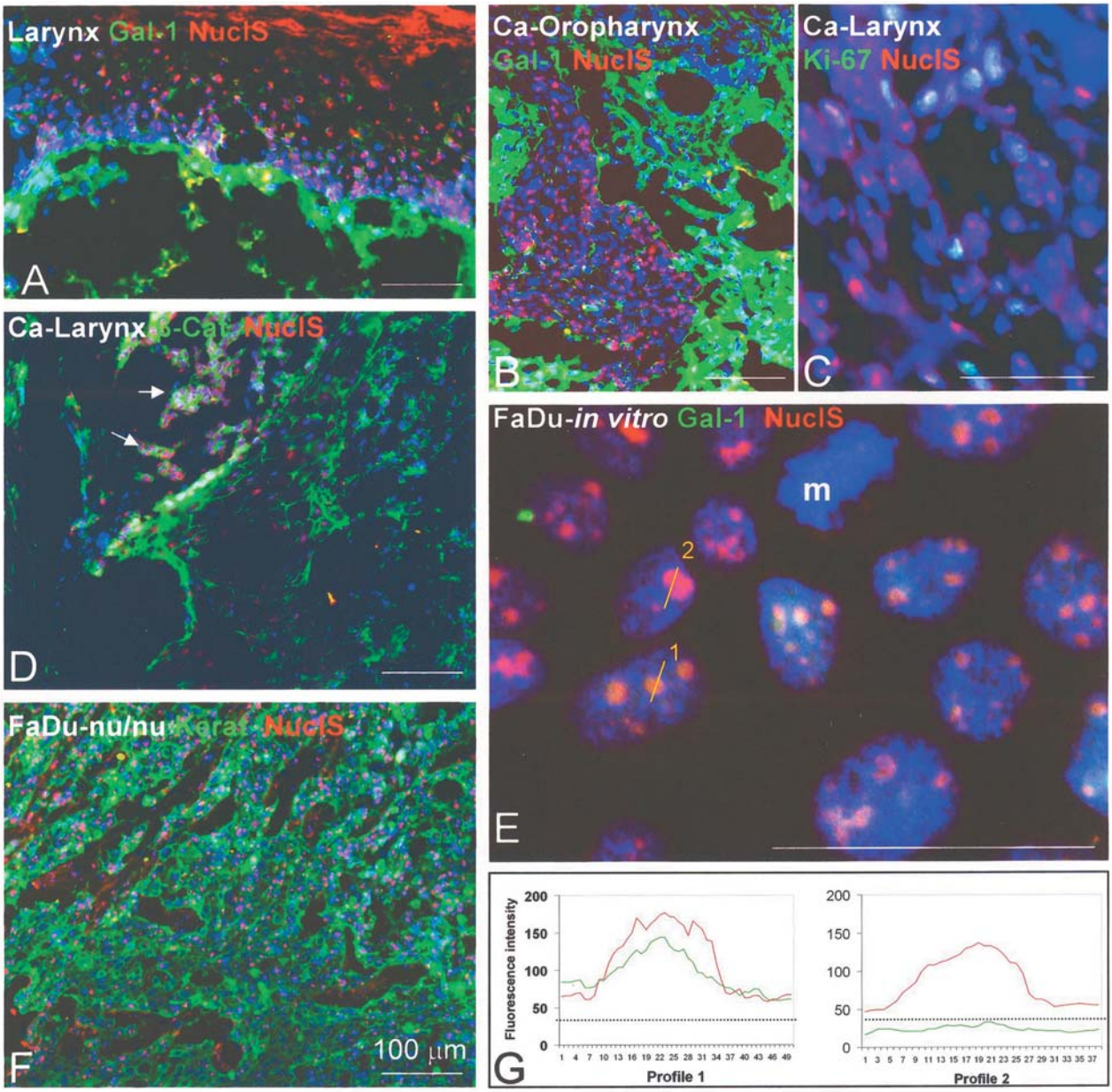

Figure 1. *Detection of nucleostemin (NuclS, red) (A-E), galectin-1 (Gal-1, green) (A, B, E), Ki-67 (green) (C), β-catenin (β-cat, green) (D) and of a pankeratin (Kerat, green) (F) in normal laryngeal epithelium (A), squamous cell carcinoma of the oropharynx (B), squamous cell carcinoma of the larynx (C, D),cultured FaDu cells (E) and in FaDu cells grown in nu/nu mice (F). All nuclei are counterstained with DAPI. Arrows indicate cells with cytoplasmic/nuclear expression of β-catenin. Mitotic cells are marked by "m". Fluorescence intensity profiles were measured for FaDu cell nucleoli marked 1 and 2 (G).*

The nuclei of cells from squamous cell carcinomas contained nucleoli which gave a strong nucleostemin signal (Figure 1B-D). Similar findings were also obtained in cultured FaDu cells (Figure 1E) and in tumors from FaDu cells grafted into mice (Figure 1F). In addition to the signal intensity the size of nucleostemin-positive nucleoli was significantly larger in cells of squamous cell carcinomas than in cells of normal epithelia (Figure 2A). This property was also detectable in FaDu cells grown both *in vivo* and *in vitro*, although it was not statistically verified (Figure 2A). Analyzing the distribution of nucleoli according to their size, the nucleolar area in normal epithelium was rather uniform with a high incidence of nucleoli in the range of 6 to 10 μm^2 (Figure 2B). In contrast, the size distribution of nucleoli in both laryngeal and oropharyngeal squamous cell carcinomas was broad, with occurrence of very large nucleoli up to 35 μm^2 (Figure

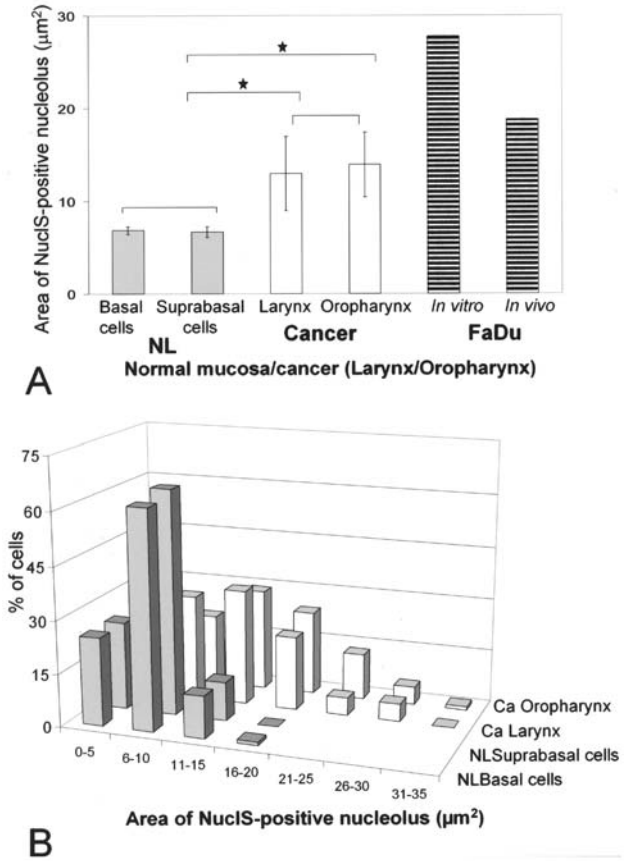

Figure 2. *A) Size of nucleostemin-positive nucleoli in basal layer cells (NL -Basal cells) of normal laryngeal epithelium, in suprabasal layer cells (NL -Suprabasal cells) of normal laryngeal epithelium, in laryngeal (Ca -Larynx) and in oropharyngeal (Ca -Oropharynx) cancer cells, in cultured FaDu cells (FaDu-in vitro), and in cells of tumors formed by grafting FaDu cells in vivo. Statistically significant differences are marked by asterisks; p=0.05. B) Size-dependent distribution of nucleostemin-positive nucleoli in basal and suprabasal layer cells, as well as in carcinoma cells of the oropharynx and larynx.*

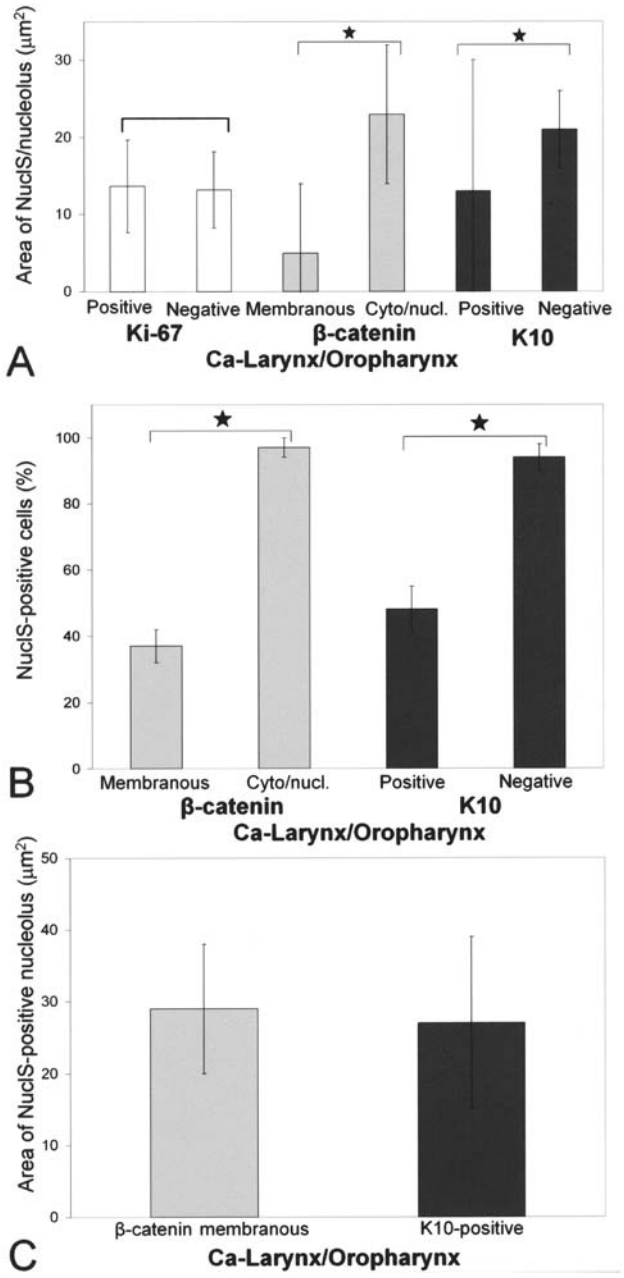

Figure 3. *A) Size of nucleostemin positivity per nucleolus in cancer cells, in relation to the expression of the proliferation marker Ki-67, the expression pattern of β-catenin (membranousscytoplasmic/nuclear) and the expression of keratin 10. B) Incidence of cancer cells according to their phenotype. C) Comparison of the size of the area expressing nucleostemin in cells positive for this marker and presenting a membrane-associated signal for β-catenin, and for keratin 10. The difference is statistically nonsignificant; p=0.05.*

2B). Having first focused on features of nucleostemin presence, we next set the immunohistochemical data in relation to proliferation and other cellular markers.The studied tumors contained groups of cells with membrane-associated signals for β-catenin with the cytoplasmic and nuclear presence of this protein (Figure 1D) that is associated with tumor progression. The mean size of the nucleostemin-positive area per nucleolus was smaller in cells with membrane-associated positivity for β-catenin than in cells with positivity in the cytoplasm/nucleus (Figure 3A). However, this result should be be considered cautiously due to the rather low degree of nucleostemin positivity in cells with membrane-associated β-catenin (Figure 1D), where approximately one half of the cells contained nucleostemin-positive nucleoli (Figure 3B). When the signal for nucleostemin was evaluated based on positive cells only, these were found to exhibit large nucleostemin-positive nucleoli (Figure 3C). Interestingly, the same phenomenon was observed for keratin 10-positive cells (Figure 3A-C).

Nuclear/nucleolar expression of galectin-1, known to be expressed in cells sharing features with epidermal stem cells was detected in cultured FaDu cells (Figure 1E). No signal for the expression of this endogenous lectin was found in the nuclei of cells from normal epithelia or carcinomas (Figure 1A, B), or in tumors from FaDu cells grown in nu/nu mice (not shown). Of note when examining the tumor sections was the abundant presence of galectin-1 in the tumor stroma (Figure 1B); its level was significantly higher than in the connective tissue of the normal mucosa (Figure 1A).

Discussion

Evidently, expression of nucleostemin is not dependent on the proliferation status of cells in squamous epithelia of either ectodermal (epidermis) or endodermal (larynx) origin, knowing that only basal cells are able to proliferate (4, 27). Similarly, the proliferation status of tumor cells has no influence on the expression of nucleostemin in their nucleoli. However, the nucleostemin-positive nucleoli are larger than these in the normal epithelia. Surprisingly, nucleostemin-positive nucleoli of a very large area were found in cancer cells exhibiting membrane attached β-catenin and keratin 10, markers indicating differentiated phenotype in the normal cells (11, 19). This finding is similar to our observation in a previous study comparing the expression of keratins, ligands for galectin and Ki-67 where difference between expression of markers of the terminal differentiation and Ki-67 can be explained by the disparity between cell maturation and differentiation in cells of squamous cell carcinomas of the head and neck (11). Galectin-1 expression in the cell nucleus and/or nucleolus was observed in cells sharing features of epidermal stem cells (24) and it was also observed in FaDu cells (10). While nucleostemin was expressed in all cultured FaDu cells nucleoli, galectin-1 was detected in one half of studied cells where the good agreement of both proteins localization was present.

Extensive expression of galectin-1 in the tumor stroma represents one of dominant features of all the studied carcinomas. Increased presence of galectin-1 in the stroma has been observed, for example, in basal cell carcinomas (28) and the dermis of psoriatic skin (29).

The presented results document the presence of nucleostemin in squamous cell carcinoma of the head and neck. A high level of expression of this nuclear protein has also been observed in brain tumors (12), basal cell carcinomas (16), stomach and liver cancers (30) and cancer of the kidney (31). By immunohistochemical means it is not possible to determine whether this high level is an inherent property of tumor cells or is induced by a crosstalk between the cancer epithelium and tumor stromal cells (28). Looking at functional aspects, nucleostemin is likely not involved in the production of rRNA (32), but it may exert other regulatory functions during malignant transformation (33). One proposed function of nucleostemin is the control of proliferation and the inhibition of senescence, a potential means by which tumor cells avoid restrictions to their growth potential also related to galectins (34-36).

Conclusion

The presence of nucleostemin was documented in head and neck cancer here, and its detection, together with the size properties of positive nucleoli, may relate to tumor cell features (37).

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References

1 Hoffman HT, Karnell LH, Funk GF, Robinson RA and Menck HR: The national cancer data base report on cancer of the head and neck. Arch Otolaryngol Head Neck Surg *124*: 951-962, 1998.

2 Chiesa F, Mauri S, Tradati N, Calabrese L, Giugliano G, Ansarin M, Andrle J, Zurrida S, Orecchia R and Scully C:. Surfing prognostic factors in head and neck cancer at the millenium. Oral Oncol *35*: 590-596, 1999.

3 Hardisson D: Molecular pathogenesis of head and neck squamous cell carcinoma. Eur Arch Otorhinolaryngol *260*: 502-508, 2003.

4 Plzak J, Smetana K Jr, Chovanec M and Betka J: Glycobiology of head and neck squamous epithelia and carcinomas. ORL *67*: 61-69, 2005.

5 Chung CH, Parker JS, Ely K, Carter J, Yi Y, Murphy BA, Murphy BA, Ang KK, El-Naggar AK, Zanation AM, Cmelak AJ, Levy S, Slebos RJ and Yarbrough WG: Gene expression profiles identify epithelial-to-mesenchymal transition and activation of nuclear factor-Î B signaling as characteristics of a high-risk head and neck squamous cell carcinoma. Cancer Res *66*: 8210-8218, 2006.

6 Reya T, Morrison SJ, Clake MF and Weissman IL: Stem cells, cancer and cancer stem cells. Nature *414*: 105-111, 2001.

7 Sell S: Stem cell origin of cancer and differentiation therapy. Crit Rev Oncol Hematol *51*: 1-28, 2004.

8 Motlık J, Klıma J, Dvorankova B and Smetana K Jr: Porcine epidermal stem cells as biomedical model for wound healing and normal/malignant epithelial cell propagation. Theriogenology *67*: 105-111, 2007.

9 Mackenzie IC: Retention of stem cell patterns in malignant cell lines. Cell Prolif *38*: 347-355, 2005.

10 Smetana K Jr, Dvorankova B, Chovanec M, Boucek J, Klıma J, Motlık J, Lensch M, Kaltner H, André S and Gabius H-J: Nuclear presence of adhesion/growth-regulatory galectins in

normal/malignant cells of squamous epithelial origin. Histochem Cell Biol *125*: 172-181, 2006.
11 Chovanec M, Smetana K Jr, Betka J, Plzak J, Brabec J, Moya-Alvarez V André S, Kodet R and Gabius H-J: Correlation of expression of nuclear protein pKi67 and p63 with lectin histochemical features in head and neck squamous cell cancer. Int J Oncol *27*: 409-415, 2005.
12 Tsai RYLand McKay RDG: A nucleolar mechanism controlling cell proliferation in stem cells and cancer cells. Genes Develop 16: 2991-3003, 2002.
13 Yaghoobi MM, Mowla SJand Tiraihi T: Nucleostemin, a coordinator of self-renewal, is expressed in rat marrow stroma cells and turns off after induction of neural differentiation. Neurosci Lett *390*: 81-86, 2005.
14 Beekman C, Nichane M, De Clerq S, Maetens M, Floss T, Wurst W Bellefroid E and Marine JC: Evolutionarily conserved role of nucleostemin: controlling proliferation of stem/progenitor cells during early vertebrate development. Mol Cell Biol *26*: 9291-9301, 2006.
15 Maki N, Takechi K, Sano S, Tarui H, Sasai Y and Agata K. Rapid accumulation of nucleostemin in nucleolus during newt regeneration. Dev Dyn in press, 2007.
16 Lacina L, Smetana K Jr, Dvorankova B, Stork J, Plzakova Z and Gabius H-J: Immunocyto- and histochemical profiling of nucleostemin expression: marker of epidermal stem cells? J Dermatol Sci *44*: 73-80, 2006.
17 Tumbar T, Guasch G, Greco V, Blanpain C, Lowry WE, Rendl M and Fuchs E: Defining the epithelial stem cell niche in the skin. Science *303*: 359-363, 2004.
18 Brown DC and Gatter KC: Ki67 protein: the immaculate deception. Histopathology *40*: 2-11, 2002.
19 Gonacci-Sorrell M, Zhurinsky J and Ben Ze'ev A: The cadherin-catenin adhesion system in signalling and cancer. J Clin Invest *109*: 987-991, 2002.
20 Plzak J, Holıkova Z, Smetana K Jr, Dvorankova B, Hercogova Z, Kaltner H and Motlık J, Gabius H-J: Differentiation-dependent glycosylation of cells in squamous cell epithelia detected by a mammalian lectin. Cells Tissues Organs *171*: 135-144, 2002.
21 Gabius H-J: Animal lectins. Eur J Biochem *243*: 543-576, 1997.
22 Gabius H-J: Cell surface glycans: the why and how of their functionality as biochemical signals in lectin-mediated information transfer. Crit Rev Immunol *26*: 43-79,2006.
23 Villalobo A, Nogales-Gonzalez A and Gabius H-J: A guide to signaling pathways connecting protein-glycan interaction with the emerging versatile effector functionality of mammalian lectins. Trends Glycosci Glycotechnol *18*: 1-37, 2006.
24 Klıma J, Smetana K Jr, Motlık J, Plzakova Z, Liu F-T, Stork J, Kaltner H, Chovanec M, Dvorankova B, André S and Gabius H-J: Comparative phenotypic characterization of keratinocytes originating from hair follicles. J Mol Histol *36*: 89-96, 2005.
25 Kayser K, Hoeft D, Hufnagl P, Caselitz J, Zick Y, André S, Kaltner H and Gabius H-J: Combined analysis of tumor growth pattern and expression of endogenous lectins as a prognostic tool in primary testicular cancer and its lung metastases. Histol Histopathol *19*: 771-779, 2003.
26 André S, Kaltner H, Furuike T, Nishimura S-I and Gabius H-J: Persubstituted cyclodextrin-based glycoclusters as inhibitors of protein-carbohydrate recognition using purified plant and mammalian lectins and wild-type and lectin-gene-transfected tumor cells as targets. Bioconjugate Chem *15*: 87-98, 2004.
27 Seery JP: Stem cells of the oesophageal epithelium. J Cell Sci *115*: 1783-1789, 2002.
28 Lacina L, Smetana K Jr, Dvorankova B, Pytlık R, Kideryova L, Kucerova L, Plzakova Z, Stork J, Gabius H-J and André S: Stromal fibroblasts from basal cell carcinoma affect phenotype of normal keratinocytes. Brit J Dermatol *156*: 819-829, 2007.
29 Lacina L, Plzakova Z, Smetana K Jr, Stork J, Kaltner H and André S: Glycophenotype of psoriatic skin. Folia Biol *52*: 10-15, 2006.
30 Liu S-J, Cai Z-W, Liu Y-J, Dong M-Y, Sun L-Q, Hu G-F, Wei Y-Y and Lao W-D: Role of nucleostemin in growth regulation of gastric cancer, liver cancer and other malignancies. World J Gastroenterol *10*: 1246-1249, 2004.
31 Fan Y, Liu Z, Zhao S, Lou F, Nilsson S, Ekman P, Xu D and Fang X: Nucleostemin mRNA is expressed in both normal and malignant renal tissues. Br J Cancer *94*: 1658-1662, 2006.
32 Ritland Politz JC, Polena E, Trask I, Bazett-Jones DP and Pederson T: A nonribosomal landscape in the nucleolus revealed by the stem cell protein nucleostemin. Mol Biol Cell *16*: 3401-3410, 2005.
33 Maggi LB and Weber JD: Nucleolar adaptation in human cancer. Cancer Invest *23*: 599-608, 2005.
34 Chovanec M, Smetana K Jr, Dvorankova B, Plzakova Z, André S and Gabius H-J: Decrease of nuclear reactivity to growth-regulatory galectin-1 in senescent human keratinocytes and detection of non-uniform staining profile alterations upon prolonged culture for galectins-1 and -3. Anat Histol Embryol *33*: 348-354, 2004.
35 Rotblat B, Niv H, André S, Kaltner H, Gabius H.-J and Kloog Y: Galectin-1 (L11A) predicted from a computed galectin-1 farnesyl-binding pocket selectivity inhibits Ras-GTP. Cancer Res *64*: 3112-3118, 2004.
36 Zhu Q, Yasumoto H and Tsai RY: Nucleostemin delays cellular senescence and negatively regulates TRF1 protein stability. Mol Cell Biol *26*: 9279-9290, 2006.
37 Smetana K: Structural features of nucleoli in blood, leukemic, lymphoma and myeloma cells. Eur J Histochem *46*: 125-132, 2002.

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Marker profiling of normal keratinocytes identifies the stroma from squamous cell carcinoma of the oral cavity as a modulatory microenvironment in co-culture

LUKAS LACINA[1,2], BARBORA DVOŘÁNKOVA[1,3], KAREL SMETANA Jr[1,3], MARTIN CHOVANEC[1,3,4], JAN PLZÁK[1,3,4], RUTH TACHEZY[5], LINDA KIDERYOVÁ[6], L. KUČEROVÁ[7], ZDENEK ČADA[1,4], JAN BOUČEK[1,8], R. KODET[9], S. ANDRÉ[10], & HANS-JOACHIM GABIUS[10]

[1]*First Faculty of Medicine, Institute of Anatomy,* [2]*First Faculty of Medicine, Department of Dermatovenerology,* [3]*Second Faculty of Medicine, Center of Cell Therapy and Tissue Repair,* [4]*First Faculty of Medicine, Department of Otorhinolaryngology, Head and Neck Surgery, all from Charles University in Prague,* [5]*Institute of Hematology and Blood Transfusion, Experimental Virology, Prague,* [6]*Charles University in Prague, First Faculty of Medicine, 1st Department of Internal Medicine,* [7]*Department of Molecular Genetics, University Hospital Hradec Kralove, Hradec Kralove, Czech Republic,* [8]*Institute of Microbiology, Academy of Science of the Czech Republic, Prague,* [9]*Charles University in Prague, Second Faculty of Medicine, Institute of Pathology and Molecular Medicine, and* [10]*Ludwig-Maximilians-University Munich, Faculty of Veterinary Medicine, Institute of Physiological Chemistry, Munich, Germany*

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Abstract

Purpose: The microenvironment established by stromal cells may or may not influence phenotypic aspects of epithelial cells and may be relevant for tumor and stem cell biology. We address this issue for keratinocytes using tumor-derived stromal cells in a co-culture system.

Materials and methods: We isolated stromal cells from human squamous cell carcinoma tissue and studied their effect on phenotypic characteristics of normal human interfollicular keratinocytes *in vitro*.

Results: Stromal fibroblasts significantly influence immuno- and lectin cytochemical properties of co-cultured normal keratinocytes. Expression of keratins 8 and 19, the nucleolar protein nucleostemin, parameters related to adhesion/growth-regulatory galectins and the epithelial-mesenchymal transition were altered. This biological activity of tumor-derived stromal cells, which did not require cell contact, appeared to be stable, because it was maintained during passaging of keratinocytes in the absence of cancer cells.

Conclusions: Tumor-derived stromal fibroblasts acquire distinct properties to shape a microenvironment conducive to altering the phenotypic characteristics of normal epithelial cells *in vitro*.

Keywords: *Cell biology, skin, stem cells, tumour physiology*

Introduction

Advances in stem cell research are likely to broaden the clinical potential of regenerative medicine, cell therapy and tissue engineering. Equally important is our understanding of stem cell biology in adult tissues, currently linked to the origin of solid tumors, which can notably factor into development of new therapeutic concepts (Reya et al. 2001, Sell 2004, Keith 2006). From this point of view, epidermal stem cells are believed to participate in the development of basal cell and squamous cell carcinomas (Owens & Watt 2003, Costea et al. 2006, Motlík et al. 2007). This hypothesis is based on functional aspects shared by normal adult stem cells and cancer cells as well as on the similarity of their phenotypes observed either under *in vitro* conditions or in biopsies. (Chovanec et al. 2005a, Dvořánkova et al. 2005, Mackenzie 2005, Smetana et al. 2006). In order to maintain their unique properties tissue stem cells require a special

Correspondence: Dr Karel Smetana, Charles University in Prague, 1st and 2nd Faculty of Medicine, Institute of Anatomy and Center of Cell Therapy and Tissue Repair, Prague, Czech Republic. E-mail: Karel.Smetana@lf1.cuni.cz

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microenvironment, whose essential biochemical components are not clearly defined as yet (Watt & Hoggan 2000). The stem cell compartment in skin is being intensively studied to address this issue (Tumbar et al. 2004). Assuming a lineage from adult stem cells to tumor cells, it is reasonable to consider the analysis of the cellular microenvironment in tumors to identify spot clues to be followed in stem cell research. Focusing on fibroblasts in the tumor stroma, an intriguing question arises as whether they contribute to the establishment of niche-like properties (Kenny & Bissell 2003, Bissell & LaBarge 2005). Of relevance are studies of the establishment of malignancy in a prostatic epithelium cell line and tumor spread after inoculation into mice, in which stroma fibroblasts appeared to favor transformation (Hayward et al. 2001). Such reports are a paradigm for the potential of tumor stromal fibroblasts to act as modulators of other cell types. This potential for modulation is the main aim of our report.

We presume that these cells are recruited from the fibroblast pool of the local mesenchyme responding to growth factors/cytokines produced by the malignant epithelium. They can also originate from the malignant epithelium by epithelial-mesenchymal transition (Petersen et al. 2003, Weber et al. 2006). A fusion of cancer epithelial cells with stroma fibroblasts was also proposed to create bioactive stroma cells (Jacobsen et al. 2006). In a previous study, we initiated monitoring of the properties of normal human keratinocytes exposed to fibroblasts derived from a human basal cell carcinoma in a co-culture system (Lacina et al. 2007). Notable changes, for example, concerned expression characteristics of keratin 19 and nucleostemin and spurred our interest to extend this line of investigation to epidermal keratinocytes under similar experimental conditions. A pertinent issue was to test stroma derived from a different tumor type, i.e., squamous cell carcinoma. Consequently, the present study focuses on monitoring phenotypic properties of normal human epidermal keratinocytes under the influence of human squamous cell carcinoma.

The stromal fibroblasts were first characterized by karyotyping, profiling of cell surface markers and immunocytochemical analysis. Keratinocytes co-cultured with the tumor-derived stromal cells, using human dermal fibroblasts and 3T3 cells as controls in parallel, were subjected to monitoring of various features relevant for differentiation and growth. Among keratins, we looked especially at keratin 8, because this type of keratin is not normally expressed in postnatal normal squamous epithelia including cultured cells and its presence in squamous cell carcinoma is an indicator of poor prognosis of patients (Gires et al. 2004). We also included keratin 19 present in epidermal stem cells, which is not normally expressed in interfollicular epidermis (Michel et al. 1996, Dvořánková et al. 2005). Keratin 19 expression can be induced in the interfollicular keratinocytes by a suspension regimen and readhesion or by co-cultivation of these cells with fibroblasts prepared from basal cell carcinoma (Dvořánková et al. 2005, Lacina et al. 2007). Although it has not been proven a reliable marker of epidermal stem cells, we added nucleostemin to our panel because this marker is expressed in keratinocytes at an early stage of differentiation under *in vitro* conditions (Lacina et al. 2006, 2007). The region of epithelial-mesenchymal transition was defined by the level of coexpression of keratins and vimentin and also by detection of snail, the transcription factor involved in control of this process (Huber et al. 2005). Due to the association of β-catenin with the course of embryogenesis and also to cancer progression the extent of its translocation from the cell membrane to either the cytoplasm or nucleus was assessed (Conacci-Sorrell et al. 2002). The same, and even in more general terms, holds true for glycan epitopes of cellular glycoconjugates acting as biochemical signals in the interplay with endogenous lectins (Gabius 1997, 2001, 2006, Buzas et al. 2006, Villalobo et al. 2006). Because expression and profiling of those ligands are of prognostic relevance in several tumor types and they are supposed to be senescence-associated indicators in keratinocytes (Chovanec et al. 2004, Gabius et al. 2004, Lahm et al. 2004, Plzák et al. 2004, Szöke et al. 2005, Smetana et al. 2006), members of the adhesion/growth-regulatory family of galectins were studied by immuno- and lectin cytochemistry. What's more, galectin expression is susceptible to modulation by microenvironmental factors including growth *in vitro* or in tumors *in vivo* (Gabius & Vehmeyer 1988). These results direct attention to a methodological factor concerning the mode of cell culture. Cell growth either on coverslips or in 3D scaffolds can also influence cell features (Smalley et al. 2006). Thus, we compared cell populations kept in the classical two-dimensional (2D) culture with those maintained in three dimensional (3D) culture in a Matrigel matrix. To delineate whether the influence of stroma cells depends either on direct contact with the epithelial cells or on paracrine mechanisms we tested the cytochemical parameters mentioned above on keratinocytes physically separated from stromal cells by a microporous membrane. The given set of experiments were flanked by two approaches to infer an ontogenetic relationship between tumor cells and the stromal cell population, i.e., (i) the application of a differentiation-promoting agent, sodium butyrate, *in vitro*, and (ii) testing of stroma in tumors obtained from cells of the human FaDu line grafted to nu/nu mice *in vivo*.

Material and methods

Characterization of tumor

Attempts were made to isolate stromal cells from squamous cell carcinomas of the head and neck of three patients but only the presented cultivation was successful. This sample was the third recurrence of a squamous cell carcinoma of the head and neck in a 60-year-old male patient. The primary tumor was located in the edge region of the base of the tongue, metastases to lung and lymph nodes were present. This patient had been treated so far surgically with subsequent radiotherapy. The dissected tumor was divided into three parts. The first part was fixed with paraformaldehyde and embedded in paraffin for routine histopathological evaluation. The second part was embedded in the cryoprotective agent Tissue-Tek (Christine Gröpl, Tulm, Austria) and frozen in liquid nitrogen. This part was used for immuno- and lectin histochemistry. The third part was used for the preparation of stroma and cancer cells for experiments *in vitro*. The entire experiment was performed by strictly obeying the policy of informed consent of patients according to the Helsinki Declaration.

Detection of human papilloma virus DNA

Head and neck squamous cell carcinomas can be etiologically linked to infection with human papilloma virus (HPV) (Smith et al. 2006). To exclude a possible influence of viral infection on the studied parameters, tumors were examined for the presence of viral DNA. After removal of paraffin with xylene, sections were incubated with proteinase K-containing solution (Sigma, Prague, Czech Republic) at a final concentration of 200 μg/ml in lysis buffer (50 mM Tris-HCl, pH 8; 5 mM EDTA [ethylenediaminetetraacetic acid]; 1% Tween 20) for 2 h at 55°C. Proteinase K was then inactivated at 95°C for 10 min, and DNA was extracted using the standard phenol/chloroform mixture and stored at −20°C. A negative control was included in the process of DNA preparation.

Detection of presence and genotyping of the HPV DNA in samples were carried out using the polymerase chain reaction (PCR) with reverse-line blot hybridization enabling genotyping of 37 different HPV types in a single assay (van den Brule et al. 2002). The HPV DNA detection was performed in a PCR thermocycler PTC 200 (MJ Research, Inc, Waltham, MA, USA) with primer GP5+ and 5′-end biotin-labeled GP6+ primer, which amplify the 150 bp-long fragment of the L1 gene. PCR was performed for 40 cycles, and the resulting biotinylated PCR product was hybridized to oligonucleotide probes labeled at the 5′-terminal amino group. These probes were covalently linked to an activated, negatively charged Biodyne C membrane. After washing, the membrane was incubated for 60 min at 42°C with peroxidase-conjugated streptavidin. Chemiluminescent detection of hybridizing DNA on the membrane used the ECL detection liquid (Amersham Biosciences, Freiburg, Germany) and exposure of the membrane to LumiFilm (Roche, Indianapolis, IN, USA) for 5 min. Detection of a fragment of the human β-globin gene was used as a internal standard. It was amplified with primers PC 03 (5′ACACAACTGTGTTCACTAGC 3′) and PC 04 (5′CAACTTCATCCACGTTCACC 3′) (Saiki et al. 1985). Positive β-globin amplification proved that the sample contained a sufficient quantity of DNA and that no inhibitors of the PCR were present. Fifty microliters of the reaction mixture contained 1× concentrated reaction buffer (Fermentas, Vilnius, Lithuania) with 4.0 mMol/l $MgCl_2$, 0.2 mMol dNTP, 0.05 pmol of each primer (PC 03 and PC 04) and 2.5 U Taq DNA polymerase (Fermentas, Vilnius, Lithuania). After an initial denaturation step for 5 min at 95°C, each of the 40 cycles comprised a 1-min period of denaturation at 95°C, primer annealing for 2 min at 55°C and chain elongation for 2 min at 72°C. In the final step, incubation for three minutes at 72°C was performed.

Cell preparation and 2D culture

Normal dermal fibroblasts (DF) and keratinocytes were prepared from skin specimens of healthy patients (with their informed consent) who underwent plastic surgery. Each skin graft was treated overnight with a 0.3% solution of trypsin at 4°C. Dermis and epidermis were separated. The tumor sample was enzymatically treated in the same way. Keratinocytes obtained from the epidermis and from the tumor samples were propagated following the modified Rheinwald-Green method (Matoušková et al. 1989). Keratinocytes from healthy donors (the first and second subcultures) were frozen in aliquots in 10% of dimethyl sulfoxide (Sigma, Praha, Czech Republic) and stored in liquid nitrogen. Fibroblast emigrating from minced dermis pieces were harvested and propagated in Dulbecco's modified Eagle's medium (DMEM) medium (Biochrom, Berlin, Germany) with 10% of fetal calf serum (Biochrom, Berlin, Germany) at 37°C and 5% CO_2. Stromal fibroblasts of the squamous cell carcinoma (SCCF) were prepared and cultured by the modified method as described (Grando et al. 1996, Lacina et al. 2007). For the experiments cells from the seventh passage cultured for 41 days, presenting a normal appearance, were used. 3T3 cells were propagated in H-MEM medium (Hanks'

salts modified Eagle's medium SevaPharma, Prague, Czech Republic) with 10% bovine serum (ZVOS, Hustopeče, Czech Republic) at 37°C and 3.3% CO_2. Prior to co-culture with keratinocytes, proliferation of 3T3 fibroblasts was impaired by exposure to mitomycin C (Sigma, Praha, Czech Republic) at a concentration of 25 μg/ml for 3 h. These cells were seeded on glass coverslides at a density of 25,000 cells/cm^2 and cultured for 24 h. Due to their rather low proliferative activity SCCF were not exposed to mitomycin C when cultured at a density of 7000 cells/cm^2. The suspension of keratinocytes (20,000 cells/cm^2) was then added, and the cells were cultivated in a keratinocyte medium (Matoušková et al. 1989) at 37°C and 3.3% CO_2. The SCCF phenotype was also studied after the treatment of cells with sodium butyrate (Sigma, Praha, Czech Republic) applied at concentrations of 0.4 or 0.8 mg/ml in culture medium as described elsewhere (Daehn et al. 2006) for three or six consecutive days.

The commercially available human hypopharyngeal squamous cell carcinoma line FaDu (HTB-43, American Type Culture Collection, Rockville, MD, USA) was cultured in modified Eagle's medium containing 10% fetal calf serum, antibiotics (100 units/ml of penicillin, 100 μg/ml of streptomycin; Sigma), 1.5 g/l $NaHCO_3$, 0.11 g/l sodium pyruvate, 0.292 g/l L-glutamine and 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid). This line was used in the animal experiment.

Cell culture in Matrigel

5×10^5 SCCF and 1×10^6 of normal keratinocytes resuspended in minimal volume (0.1 ml) and mixed with 1.5 ml of BD Matrigel^{TM} (BD Biosciences, Erembodegen, Belgium) were placed in a culture dish (3.5 cm). This 3D system was kept under conditions as described above for seven days. After this period, the Matrigel specimen with the cells was frozen in liquid nitrogen and prepared for histochemical analysis.

Grafting of FaDu cells to nu/nu mice

Two female nu/nu CD-1 mice, aged ten weeks, were purchased from the Institute of Molecular Genetics, Academy of Science of the Czech Republic (Prague, Czech Republic). They were kept in accordance to approved guidelines and had access to food and water *ad libitum*. 1×10^6 FaDu cells were resuspended in 100 μl of phosphate-buffered saline (PBS; pH7.3) and mixed with 50 μl of BD Matrigel^{TM} (BD Biosciences, Erembodegen, Belgium) as described. This suspension was subcutaneously injected, the animals were sacrificed after 49 days and tumor specimens were frozen as described above.

Cytogenetic analysis

SCCF from the 9th passage were subcultured for 24 h, incubated with demecolcemid (Sigma) for 4 h, detached from the substratum by applying trypsin-EDTA solution, then treated by hypotonic KCl solution and fixed by acidic ethanol. Metaphasic chromosomes were analyzed after G-/R-banding using the Ikaros version 5 (MetaSystems, Altlussheim, Germany). A total of 50 metaphases were monitored in the samples investigated.

FACS analysis of DF and SCCF

DF after a brief culture period and SCCF cultured for 41 (9th passage) days were harvested using trypsin-EDTA solution. The activity of trypsin was neutralized by adding fetal calf serum. Cells were then resuspended in fresh culture medium and analyzed for presence of the following markers: cluster of differentiation (CD)11b, CD18, CD29, CD44, CD45, CD49a, CD49d, CD63, CD90, CD106, and CD166 (all from Becton Dickinson, Prague, Czech Republic), CD11c, CD14, CD34, CD45, CD71, CD235a, CD105, and HLA-A, -B, and -C (all from Dako, Brno, Czech Republic), CXCR4 (R&D Systems, Minneapolis, MN, USA) as well as CD19e and CD49c (Chemicon, Temecula, CA, USA). IgG_1 (Dako, Brno, Czech Republic) was used as a negative control. Measurements were performed on a FACSCalibur® instrument (BD Biosciences Immunocytometry Systems, San Jose, CA, USA) and analyzed using Summit® V3.3. Build 1024 software (Dako, Brno, Czech Republic).

Immuno- and lectin cytochemistry

The seven μm-thick frozen sections from human/mouse tumors and cell-containing Matrigel samples, as well as cells adherent to coverslips, were washed with PBS and briefly fixed with 5% paraformaldehyde diluted in PBS. The human galectins were purified by affinity chromatography as crucial step after recombinant production. Purity was ascertained prior to use as antigen and the resulting polyclonal antibody preparations were subjected to rigorous specificity controls including chromatographic removal of cross-reactive material, if necessary (André et al. 1999, 2004, Kayser et al. 2003a, 2003b, Kopitz et al. 2003, Dam et al. 2005, Lensch et al. 2006). Biotinylation was performed under activity-preserving conditions. Activity was ascertained by solid-phase and cell-binding assays and extent of labeling quantitated by a proteomics protocol (Gabius et al. 1984, Purkrábková et al. 2003, André et al. 2005a, 2005b, 2006, Wu et al. 2006). Staining was visualized as described previously (Froňková

et al. 1999, Plzák et al. 2001). The entire panel of monoclonal/polyclonal antibodies and the biotinylated galectins is shown in Table I. The antigen-dependent specificity was tested by replacement of the test antibody with another polyclonal or monoclonal antibody of the same isotype but against antigens not present in the studied cells/tissues. Ligand-dependent binding of galectins was tested by omission of galectin (and using the second-step reagent only) and by use of lactose to block carbohydrate-dependent binding. Nuclei of the majority of specimens were counterstained with DAPI (4′,6′-diamidino-2-phenyindole dilactate, Sigma), a DNA-specific dye. The processed specimens were finally mounted to Vectashield (Vector Laboratories, Bourlingame, CA, USA), inspected and analyzed using an Eclipse 90i fluorescence microscope (Nikon, Prague, Czech Republic) equipped with suitable filterblocks, a high resolution Vosskühler Cool-1300Q CCD camera (Vosskühler, Osnabrück, Germany) and a computer-assisted image analyzer (LUCIA 5.10) (Laboratory Imaging, Prague, Czech Republic). Statistical calculations using the Student's non-paired *t*-test was used to assess significance levels.

Results

The investigated stroma in this study originated from a well-differentiated keratinizing squamous cell carcinoma with keratin pearls containing keratin 10-positive cells (Figure 1). In addition, keratin 8 presence was also observed (Figure 1). Intensity of staining for galectin-7, a marker of squamous epithelium, was only weak, and keratinized tumor parts were typically reactive with galectin-3 (not shown). We performed PCR analysis to exclude the confounding influence of HPV infection. No HPV-specific DNA was found in the tumor sample (Figure 2). Next, we also checked for occurrence of karyotype abnormalities. Comparing the karyotype of SCCF with normal human dermal fibroblasts, 80% of studied cells exhibited no differences (Figure 3). Absence of the Y chromosome was observed in 20% of studied cells (not shown). As a further comparative measure we monitored a panel of the surface markers using FACS analysis. No significant differences were observed between SCCF and normal DF including the absence of CD34 and CD105 (Figure 3). Then we proceeded to immunocytochemical monitoring.

SCCF were strongly positive for vimentin (Vim, Figure 4A) and devoid of keratin expression (K, Figure 4B). They exhibited high proliferative activity as demonstrated by detection of the proliferation marker Ki67, predominantly in nucleoli of approximately 30% of the studied cell population (Figure 4C, 4D). Nucleoli were strongly positive for nucleostemin that was detected in both the Ki67-positive and -negative nuclei (NuclS, Figure 4C). Approximately 20% of the SCCF expressed the adhesion/growth-regulatory galectin-1 in the cytoplasm, and the presence of this endogenous lectin was also detected in the extracellular matrix produced by these cells (Gal-1, Figure 4D). Using this lectin as a probe, nuclei of SCCF were positive as well as nuclei of malignant epithelial cells isolated from the tumor (Figure 4E). These malignant

Table I. Probes used for phenotypic characterization of cells.

Visualized epitope	Type of probe	Supplier/origin	Second-step reagent	Supplier
Panel of keratins (K1 = LP34) Keratin 8 Keratin 10 Keratin 19 Ki67 Vimentin (clone V9)	mMA	Dako, Brno, Czech Republic	a) SwAM-FITC b) Goat anti-mouse IgG-TRITC	a) AlSeVa, Prague, Czech Republic b) Sigma, Prague, Czech Republic
Panel of keratins Snail	rPA	Abcam, Cambridge, UK	SwAR-FITC	AlSeVa, Prague, Czech Republic
β-Catenin	rPA	Santa Cruz, Santa Cruz, CA, USA		
Nucleostemin	gPA	Neuromics, Bloomington, MN, USA	Donkey anti-goat-TRITC	Jackson Laboratories, West Grove, PA, USA
Galectin-1 Galectin-3 Galectin-7	rPA	Munich lab	SwAR-FITC	AlSeVa
Galectin-1-binding sites Galectin-3-binding sites	Biotinylated lectin	Munich lab	ExtrAvidin-TRITC	Sigma

mMA, mouse monoclonal antibody; gPA, goat polyclonal antibody; rPA, rabbit polyclonal antibody; SwAM-FITC, FITC-labeled swine anti-mouse antibody; SwAR-FITC, FITC-labeled swine anti-rabbit antibody.

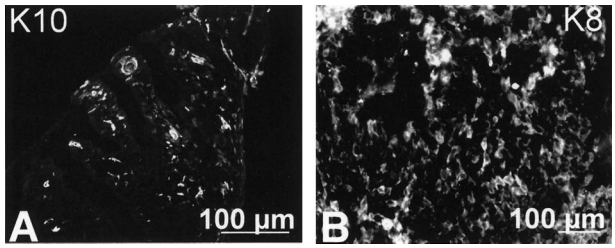

Figure 1. Immunohistochemical detection of keratin 10 (A) and keratin 8 (B) in a well differentiated keratinizing squamous cell carcinoma from which the stromal cells were prepared; scale bar: 100 μm.

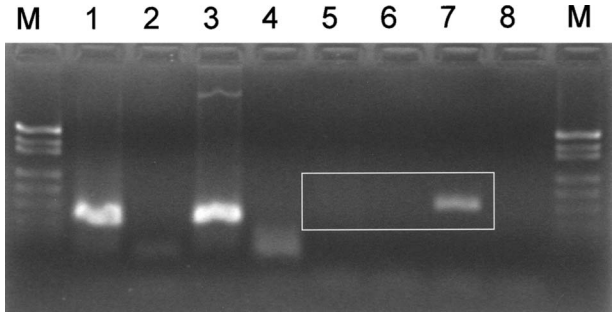

Figure 2. Search for HPV-specific DNA in the tumor by PCR. Lane M: marker; lane 1: section of β-globin gene amplified from patient material; lane 2: negative control for specific amplification of the β-globin gene; lane 3: positive control for amplification of the β-globin gene; lane 4: H_2O control; lane 5: processing of DNA sample from the tumor to detect HPV presence; lane 6: negative control of amplification of the HPV-specific DNA; lane 7: positive control of amplification of the HPV-specific DNA (type 16); lane 8: H_2O control. The rectangle denotes the region for the expected amplification product of HPV DNA, showing no signal in the sample prepared from the characterized tumor.

keratinocytes expressed keratin 8 (K8, Figure 4E), as was also observed in tissue sections. Interfollicular epidermal keratinocytes co-cultured with dermal fibroblasts or with 3T3 cells were characterized by their characteristically round morphology (Figure 4F, 4K, 4M) that contrasted with irregular shape of colonies cultured in the presence of SCCF (Figure 4L, 4N). Moreover, their phenotype was also significantly changed. While keratinocytes cultured with non-tumor stromal fibroblasts were negative for expression of keratin 8 (Figure 4F), the introduction of SCCF induced expression of this type of keratin, normally not present in the postnatal squamous epithelia (Figure 4G). Keratinocytes strongly positive for keratin 8 were observed under the influence of SCCF, mainly on the periphery of colonies (Figure 4G). These keratin 8-positive cells contained nucleoli with nucleostemin (Figure 4H). When we compared the influence of SCCF without/with treatment of mitomycin on normal keratinocytes, the keratinocytes grown together with growth-arrested SCCF were more spread than those cocultured with untreated SCCF (Figure 4H, 4I and Figure 5). Nucleostemin was detected in normal keratinocytes only if they were co-cultured with SCCF not pretreated by mitomycin C (Figure 4H). Evidently, the proliferative activity of SCCF has impact on its modulatory role. In full accordance to our previous report (Lacina et al. 2007) the stem-cell-characteristic expression of keratin 19 was observed only in keratinocytes co-cultured with SCCF (Figure 4J). Concerning the expression pattern of β-catenin, the presence of SCCF in the culture led to an intracellular shift of this protein from the cell membrane (Figure 4K) to the cytoplasm and nucleus (Figure 4L). Normal keratinocytes express keratins as a cell-type-specific form of intermediate filament, whereas the presence of vimentin is typical for fibroblasts. We visualized both cytoskeletal elements simultaneously in order to map the epithelial-mesenchymal transition. Interfollicular epidermal keratinocytes expressed keratins only (Figure 4M) when cocultured with DF and 3T3 cells, respectively. The presence of SCCF altered this expression pattern, and both types of protein, i.e. keratins and vimentin, were detected in keratinocytes, namely in cells with elongated fibroblast-like morphology (Figure 4N). Moreover, the presence of the transcription factor snail, a mediator in the control of the epithelial-mesenchymal transition, was observed in elongated epithelial cells expressing keratins (Figure 4O).

In principle, the documented influence of SCCF on interfollicular epidermal keratinocytes can be mediated by two mechanisms, i.e., by intercellular contacts or by paracrine supply of growth factors/cytokines produced by SCCF. To resolve this issue, we cultured epidermal cells, now separated from the SCCF by a microporous membrane inside the insert system. These keratinocytes also expressed keratin 8 (Figure 6A1), and cells with dual positivity for keratins and for vimentin (Figure 4P, P1) were identified in the pool of keratinocytes.

Interestingly, nucleostemin-positive nucleoli were significantly larger in keratin 8-positive than in keratin 8-negative cells (Figure $6A_{1-4}$). When considering culture methods an important aspect to be reckoned with is the mode of cell maintenance.

It is known that 2D/3D cell culture systems yield non-uniform results. Thus, we also cultivated normal interfollicular keratinocytes with SCCF in Matrigel. Whereas keratinocytes formed distinct spheroids, fibroblasts-like cells were located in their periphery (Figure 4Q). Cells in such spheres expressed keratin 8 (Figure 4R). These keratinocytes inside the spheres and cells with fibroblast-like shape expressed vimentin (Figure 4S, S_1, S_2). A rather high extent of epithelial-mesenchymal transition induced by SCCF is thus revealed as noted in the 2D system. So far, we focused on testing of SCCF as the source

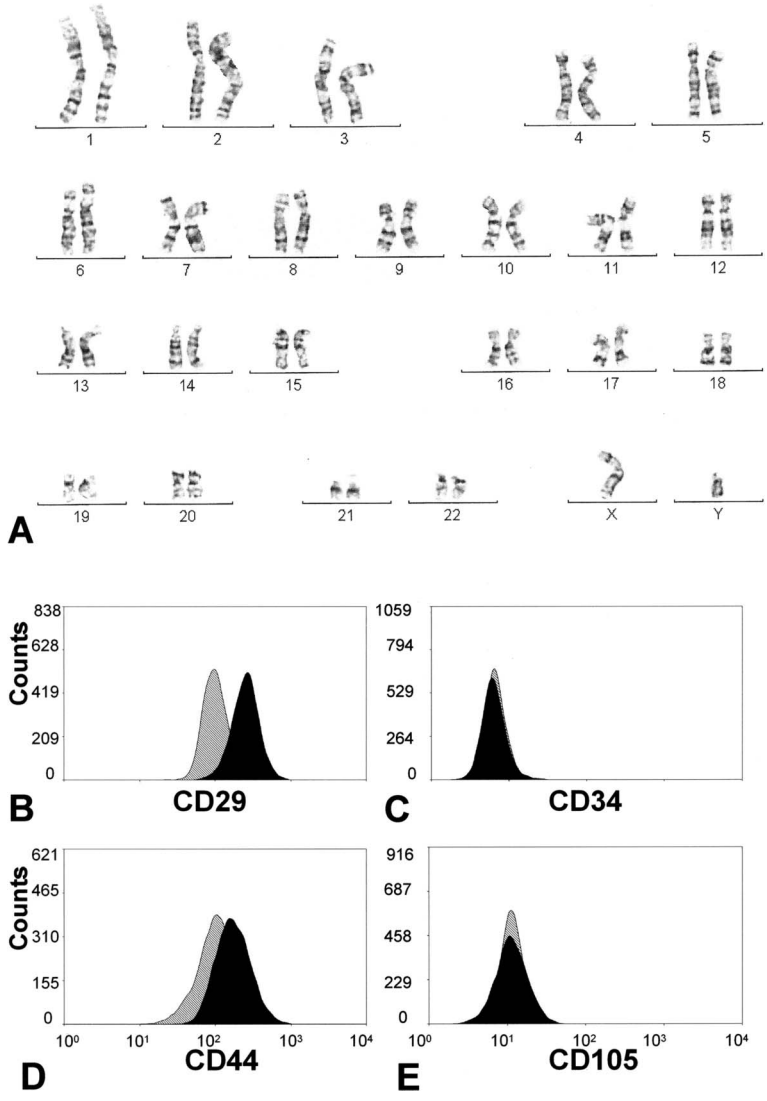

Figure 3. Karyotype of representative stromal fibroblast (A) and comparison of FACScan profiles of SCCF (black) and dermal fibroblasts (stiped). Results for CD29 (B), CD34 (C), CD44 (D) and CD105 (E) in both cell populations is presented.

for modulatory effects. To provide information on the origin of the SCCF we challenged the concept of an epithelial-mesenchymal transition by two separate experimental designs.

First, we exposed SCCF to sodium butyrate to revert the phenotype. Very few cells with keratins in addition to vimentin were present in the population of SCCF (Figure 4U), even after six days. Butyrate presence thus led to no major occurrence of reversion to epithelial cells. Should SCCF have arisen from a transition process from the pool of cancer cells, a marked extent of this process would be expected. Along this line, an *in vivo* experiment with xenotransplantation provided no evidence for such a transition. FaDu tumor cells developed large tumors in mice so that we could probe the species origin of tumor stroma. When using an antibody specific for human and porcine but not reactive with the mouse protein, no signal for vimentin expression was observed (Figure 4V). In the tested tumors stromal cells are apparently of murine nature, not a product of a transition from human tumor cells.

Discussion

We have previously initiated a study of the modulatory influence of stromal cells from a basal cell carcinoma on normal epidermal cells (Lacina et al. 2007). To extend the experimental basis of this study and to test stromal cells from squamous cell carcinoma we carried out a corresponding study. To exclude a potentially confounding factor the tumor material, which was also carefully karyotyped, was rigorously examined for the absence of HPV infection by PCR. One of the main findings of our report is the expression of keratin 8 under the influence of SCCF. This keratin is postnatally expressed in monolayer epithelia, not in squamous

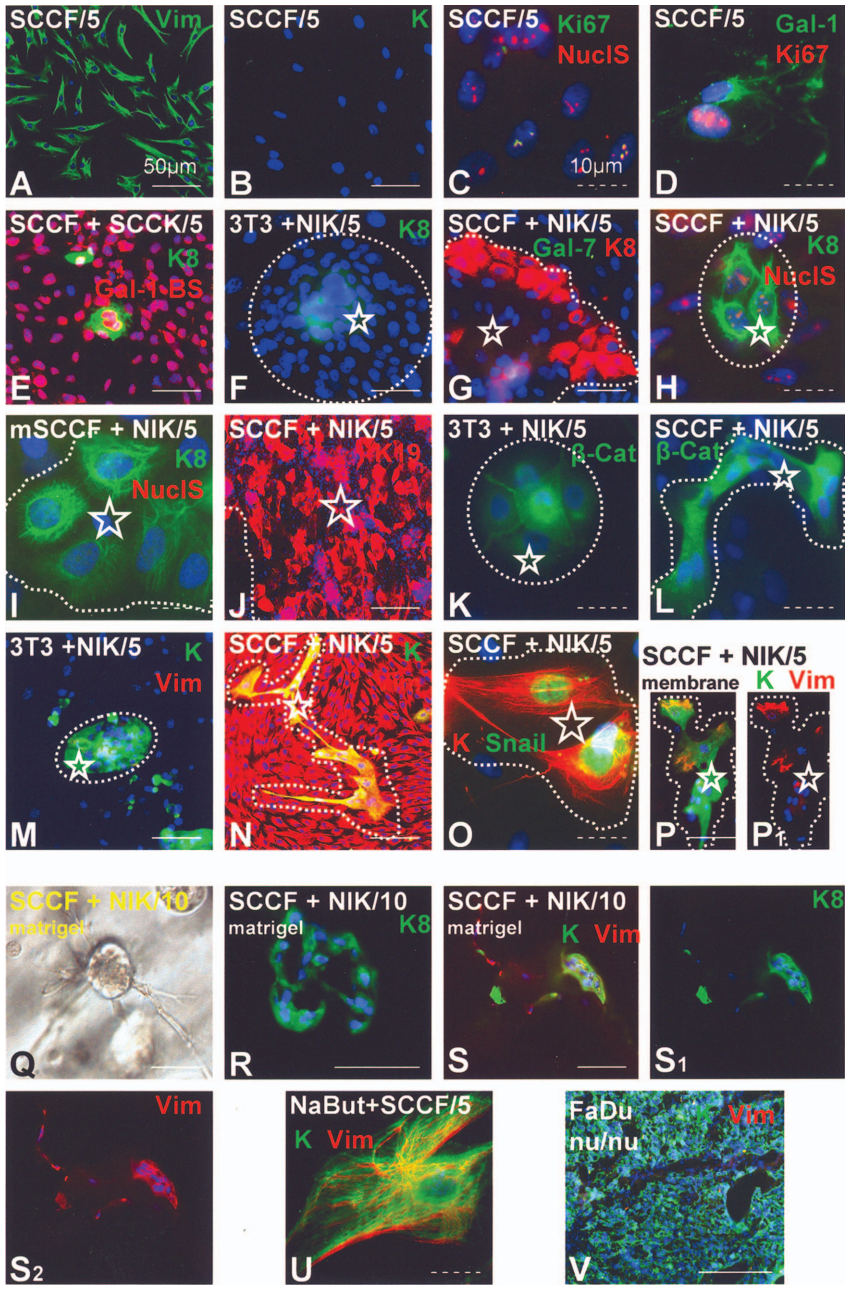

Figure 4. Immuno- and lectin cytochemical profiling of the phenotype of cultured SCCF (A-D), of cultured SCCF treated with sodium butyrate (NaBut, U), of cancer keratinocytes (SCCK) cocultured with SCCF (E), of normal interfollicular keratinocytes (NIK/5) co-cultured with 3T3 cells (F, K, M), of NIK/5 co-cultured with SCCF on coverslips (G-J, L, N, O), of NIK/5 separated from SCCF by a microporous membrane (P, P1-detection of vimentin, Vim, only), NIK co-cultured with SCCF in Matrigel (Q-S_2) and of FaDu cells in a tumor formed after xenotransplantation to nu/nu mice (V). Normal interfollicular keratinocytes were co-cultured with stromal cells or with 3T3 fibroblasts for 5 or 10 days (NIK/5, NIK/10). The applied markers are directly defined in each figure, the respective name given in the color of the detected signal. Yellow signal in panel N arises from merging red and green signals. Abbreviations: Vim, vimentin; K, panel of keratins; NuclS, nucleostemin; Gal-1, galectin-1; Gal-1-BS, binding sites for galectin-1; K8, keratin 8; Gal-7, galectin-7, K19, keratin 19; β-Cat, β-catenin. Solid Bar: 50 μm, dashed bar: 10 μm. The keratinocytes cocultured with fibroblasts on the surface of coverslips without matrigel are surrounded by the white dashed line and marked by star.

cell epithelium under physiological conditions. Moreover, keratin 8 is expressed in embryoid bodies originating from embryonic stem cells, these cells probably being precursors of epidermal stem cells (Troy & Turksen 2005). Its overexpression is associated with the malignant phenotype (Casanova et al. 2004, Raul et al. 2004). Fittingly, the increased expression correlated with poor clinical prognosis in head and neck cancer patients (Gires et al. 2004). We detected expression of this keratin in the tumor from which the stromal cells were prepared. In line with this result, nucleostemin expression is enhanced

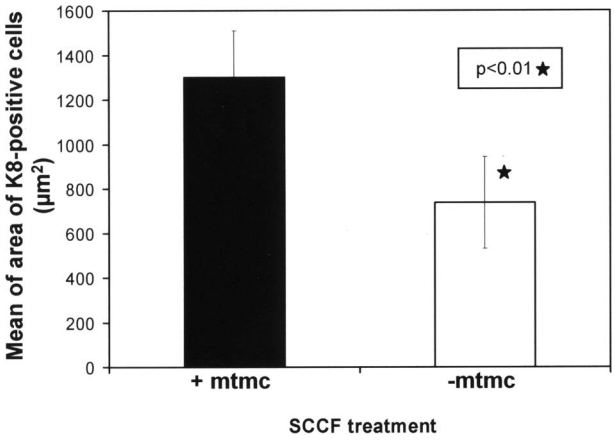

Figure 5. Comparison of the size (mean area) of normal keratinocytes (mean ± SD) cocultured with SCCF plus or minus exposure to mitomycin (mtmc), 25 μg/ml for three hours (black column: presence of mitomycin C (mtmc); white column: no mtmc). The difference is statistically significant ($p \leq 0.01$).

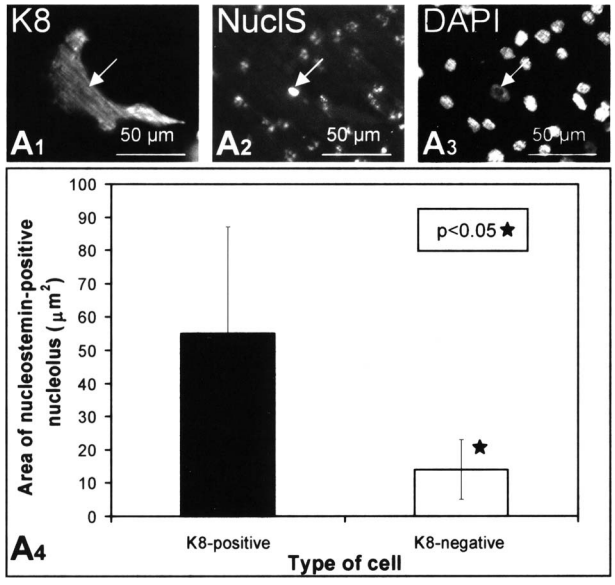

Figure 6. SCCF induced expression of keratin 8 in normal keratinocytes separated by use of a microporous membrane (A_1-$_3$). These keratin 8-positive cells contained nucleostemin (NuclS) positive nucleoli significantly larger than those of the keratin 8-negative cells (mean ± SD) (A_4). The difference is statistically significant ($p \leq 0.05$).

in normal keratinocytes cultured with fibroblasts prepared from the stroma of squamous cell carcinoma. Nucleostemin expression is involved in cancer growth regulation (Liu et al. 2004, Tsai & McKay 2002, Lacina et al. 2006) and previously was reported to be up-regulated under the influence of basal-cell-carcinoma-derived stromal cells (Lacina et al. 2007). A shift of the signal for β-catenin from association with the cell membrane to the cytoplasm/nucleus can also be considered as 'protumoral' (Conacci-Sorrell et al. 2002), and, indeed, it was observed in keratinocytes cultured with SCCF. Next, monitoring of the epithelial-mesenchymal transition based on induction of vimentin and snail expression in keratin-positive keratinocytes in both the 2D and 3D culture systems revealed a clear effect of SCCF on the transition. This is consistent with the fact that it is widely accepted that vimentin is marker of mesenchymal cells and keratins are specific for epithelial cells (Petersen et al. 2003). The epithelial-mesenchymal transition is performed under the snail control (Thiery & Sleeman 2006). This process is characteristic for embryonic development and wound healing. In malignancy it represents a high-risk factor relevant for tumor spread of cancer cells (Takkunem et al. 2006, Thiery & Sleeman 2006). The absence of galectin-7, which was studied in this tumor type previously (Chovanec et al. 2005b), together with binding of galectin-1 to cell nuclei, characteristic of cells with low level of differentiation (Klíma et al. 2005), added to the accumulating evidence of the strong modulation of keratinocyte properties. As a means to gauge the importance of intercellular contacts for this effect we introduced a membrane into the co-culture system providing physical separation of the two cell populations but allowing communication mediated via diffusible biochemical compounds. This arrangement did not impair the biological effect of SCCF on normal keratinocytes. Paracrine mediators thus appear sufficient to trigger changes in the monitored phenotypic characteristics. The production of growth-stimulatory/proangiogenic factors has been similarly discussed for inflammatory cells in tumors (Ichim 2005). Regarding the origin of the stromal cells, our results provide no evidence for cell fusion or an epithelial-mesenchymal transition process. In other words, local fibroblasts of the tumor have acquired special properties to affect phenotypic characteristics of keratinocytes *in vitro*.

In conclusion, stromal cells of squamous cell carcinoma are capable of altering keratinocyte properties in co-culture in a characteristic manner, namely, enhancement of keratin 8 expression and the epithelial-mesenchymal transition, among other parameters. This may even signify clinical relevance, e.g., for development of resistance mechanisms against radiotherapy (Smith & Haffty 1999, Diehn & Clarke 2006). The origin of stromal cell plasticity and the biochemical mechanisms underlying the effect on epithelial cells including tissue stem cells warrant further study.

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References

André S, Kojima S, Yamazaki N, Fink C, Kaltner H, Kayser K, Gabius H-J. 1999. Galectins-1 and -3 and their ligands in tumor biology. Journal of Cancer Research and Clinical Oncology 125:461–474.

André S, Kaltner H, Furuike T, Nishimura S-I, Gabius H-J. 2004. Persubstituted cyclodextrin-based glycoclusters as inhibitors of protein-carbohydrate recognition using purified plant and mammalian lectins and wild-type and lectin-gene-transfected tumor cells as targets. Bioconjugate Chemistry 15:87–98.

André S, Kojima S, Prahl I, Lensch M, Unverzagt C, Gabius H-J. 2005a. Introduction of extended LEC-14-type branching into core-fucosylated biantennary N-glycan. Glycoengineering for enhanced cell binding and serum clearance of the neoglycoprotein. FEBS Journal 272:1986–1998.

André S, Siebert H-C, Nishiguchi M, Tazaki K, Gabius H-J. 2005b. Evidence for lectin activity of a plant recetor-like protein kinase by application of neoglycoproteins and bioinformatic algorithms. Biochim Biophys Acta 1725:222–232.

André S, Pei Z, Siebert H-C, Ramström O, Gabius H-J. 2006. Glycosyldisulfides from dynamic combinatorial libraries as O-glycoside mimetics for plant and mammalian lectins: Their reactivities in soldi-phase and cell assays and conformational analysis by molecular dynamics simulations. Bioorganic & Medical Chemistry 14:6314–6326.

Bissell MJ, LaBarge MA. 2005. Context, tissue plasticity, and cancer: Are tumor stem cells also regulated by the microenvironment? Cancer Cell 7:17–23.

Buzas EI, György B, Páztói M, Jelinek I, Falus A, Gabius H-J. 2006. Carbohydrate recognition systems in autoimmunity. Autoimmunity 39:691–704.

Casanova ML, Bravo A, Martinez-Palacio J, Fernandez-Acenero MJ, Villanueva C, Larcher F, Conti CJ, Jorcano JL. 2004. Epidermal abnormalities and increased malignancy of skin tumors in human epidermal keratin 8-expressing transgenic mice. FASEB Journal 18:1556–1558.

Chovanec M, Smetana K Jr, Dvořánková B, Plzákova Z, André S, Gabius H-J. 2004. Decrease of nuclear reactivity to growth-regulatory galectin-1 in senescent human keratinocytes and detection of non-uniform staining profile alterations upon prolonged culture for galectins-1 and -3. Anatomy Histology Embryology 33:348–354.

Chovanec M, Smetana K Jr, Betka J, Plzák J, Brabec J, Moya-Álvarez V, André S, Kodet R, Gabius H-J. 2005a. Correlation of expression of nuclear protein pKi67 and p63 with lectin histochemical features in head and neck squamous cell cancer. International Journal of Oncology 27:409–415.

Chovanec M, Smetana K Jr, Plzák J, Betka J, Plzáková Z, Štork J, Hrdličková E, Kuwabara I, Dvořánková B, Liu F-T, Kaltner H, André S, Gabius H-J. 2005b. Detection of new diagnostic markers in pathology by focus on growth-regulatory endogenous lectins. The case study of galectin-7 in squamous epithelia. Prague Medical Report 106:209–216.

Costea DE, Tsinkalovski O, Vintermyr OK, Johannessen AC, Mackenzie IC. 2006. Cancer stem cells: New and potentially important targets for the therapy of oral squamous cell carcinoma. Oral Disease 12:443–454.

Daehn IS, Varelias A, Rayner TE. 2006. Sodium butyrate induced keratinocyte apoptosis. Apoptosis 11:1379–1390.

Dam TK, Gabius H-J, André S, Kaltner H, Lensch M, Brewer CF. 2005. Galectins bind to multivalent glycoprotein asialofetuin with enhanced affinities and a gradient of decreasing binding constants. Biochemistry 44:12564–12571.

Diehn M, Clarke MF. 2006. Cancer stem cells and radiotherapy: New insights into tumor radioresistance. Journal of National Cancer Institute 98:1755–1757.

Dvořánková B, Smetana K Jr, Chovanec M, Lacina L, Štork J, Plzáková Z, Galovičová M, Gabius H-J. 2005. Transient expression of keratin 19 is induced in originally negative interfollicular epidermal cells by adhesion of suspended cells. International Journal of Molecular Medicine 16:525–531.

Froňková V, Holíková Z, Liu F-T, Homolka J, Rijken DC, André S, Bovin NV, Smetana K Jr, Gabius H-J. 1999. Simultaneous detection of endogenous lectins and their binding capability at the single-cell level: A technical note. Folia Biologica (Praha) 45:157–162.

Gabius H-J. 1997. Animal lectins. European Journal of Biochemistry 243:543–576.

Gabius H-J. 2001. Glycohistochemistry: the why and how of detection and localization of endogenous lectins. Anatomy Histology Embryology 30:3–31.

Gabius H-J. 2006. Cell surface glycans: The why and how of their functionality as biochemical signals in lectin-mediated information transfer. Critical Reviews in Immunology 26:43–79.

Gabius H-J, Vehmeyer K. 1988. Effect of microenvironment and cell-line type on carbohydrate-binding proteins of macrophage-like cells. Biochemistry and Cell Biology 66:1169–1176.

Gabius H-J, Engelhardt R, Rehm S, Cramer F. 1984. Biochemical characterization of endogenous carbohydrate-binding proteins from spontaneous murine rhabdomyosarcoma, mammary adenocarcinoma, and ovarian teratoma. Journal of National Cancer Institute 73:1349–1357.

Gabius H-J, Siebert H-C, André S, Jiménez-Barbero J, Rüdiger H. 2004. Chemical biology of the sugar code. ChemBioChem 5:740–764.

Gires O, Münz M, Schaffrik M, Kleu C, Rauch J, Ahlemann M, Eberle D, Mack B, Wolleneberg B, Lang S, Hofmann T, Hammerschmidt W, Zeidler R. 2004. Profile identification of disease-associated humoral antigens using AMIDA, a novel proteomics-based technology. Cellular and Molecular Life Sciences 61:1198–1207.

Conacci-Sorrell M, Zhurinsky J, Ben-Ze'ev A. 2002. The cadherin-catenin adhesion system in signalling and cancer. Journal of Clinical Investigation 109:987–991.

Grando SA, Schofield O, Skubitz AP, Kist DA, Zelickson BD, Zachary CB. 1996. Nodular basal cell carcinoma *in vivo* vs. *in vitro*. Archives of Dermatology 132:1185–1193.

Hayward SW, Wang Y, Cao M, Hom YK, Zhang B, Grossfeld GD, Sudilovsky D, Cunha GR. 2001. Malignant transformation in a nontumorigenic human prostatic epithelial cell line. Cancer Research 61:8135–8142.

Huber MA, Kraut N, Beug H. 2005. Molecular requirements for epithelial-mesenchymal transition during tumor progression. Current Opinion in Cell Biology 17:548–558.

Ichim CV. 2005. Revisiting immunosurveillance and immunostimulation: implications for cancer immunotherapy (review). Journal of Translational Medicine 3:8.

Jacobsen BM, Harrell JC, Jedlicka P, Borges VF, Vardella-Garcia M, Horwitz KB. 2006. Spontaneous fusion with, and transformation of mouse stroma by, malignant human breast cancer epithelium. Cancer Research 66:8274–8279.

Kayser K, Hoeft D, Hufnagl P, Caseliz J, Zick Y, André S, Kaltner H, Gabius H-J. 2003a. Combined analysis of tumor growth pattern and expression of endogenous lectins as a prognostic tool in primary testicular cancer and its lung metastases. Histology and Histopathology 18:771–779.

Kayser K, Nwoye JO, Kosjerina S, Goldmann T, Vollmer E, Kaltner H, André S, Gabius H-J. 2003b. Atypical adenomatous hyperplasia of lung: Its incidence and analysis of clinical, glycohistochemical and structural features including newly defined growth regulators and vascularization. Lung Cancer 42:171–182.

Keith WN. 2006. Cancer stem cells: Opportunities for novel diagnostics and drug discovery. European Journal of Cancer 42:1195–1196.

Kenny PA, Bissell MJ. 2003. Tumor reversion: Correction of malignant behavior by microenvironmental cues. International Journal of Cancer 107:688–695.

Klíma J, Smetana K Jr, Motlík J, Plzáková Z, Liu F-T, Štork J, Kaltner H, Chovanec M, Dvořánková B, André S, Gabius H-J. 2005. Comparative phenotypic characterization of keratinocytes originating from hair follicles. Journal of Molecular Histology 36:89–96.

Kopitz J, André S, von Reitzenstein C, Versluis K, Kaltner H, Pieters RJ, Wasano K, Kuwabara I, Liu F-T, Cantz M, Heck AJR, Gabius H-J. 2003. Homodimeric galectin-7 (p53-induced gene 1) is a negative regulator for human neuroblastoma cells. Oncogene 22:6277–6288.

Lacina L, Smetana K Jr, Dvořánková B, Štork J, Plzáková Z, Gabius H-J. 2006. Immunocyto- and histochemical profiling of nucleostemin expression: Marker of epidermal stem cells? Journal of Dermatological Sciences 44:73–80.

Lacina L, Smetana K Jr, Dvoranková B, Pytlik R, Kideryova L, Kucerova L, Plzakova Z, Stork J, Gabius H-J, André S. 2007. Stromal fibroblasts from basal cell carcinoma affect phenotype of normal keratinocytes. British Journal of Dermatology 156:819–829.

Lahm H, André S, Hoeflich A, Kaltner H, Siebert H-C, Sordat B, von der Lieth C-W, Wolf E, Gabius H-J. 2004. Tumor galectinology: Insights into the complex network of a family of endogenous lectins. Glycoconjugate Journal 20:227–238.

Lensch M, Lohr M, Russwurm R, Vidal M, Kaltner H, André S, Gabius H-J. 2006. Unique sequence and expression profiles of rat galectins-5 and -9 as a result of species-specific gene divergence. International Journal of Biochemistry & Cell Biology 38:1741–1758.

Liu S-J, Cai Z-W, Liu Y-J, Dong M-Y, Sun L-Q, Hu G-F, Wei Y-Y, Lao W-D. 2004. Role of nucleostemin in growth regulation of gastric cancer, liver cancer and other malignancies. World Journal of Gastroenterology 10:1246–1249.

Mackenzie IC. 2005. Retention of stem cell patterns in malignant cell lines. Cell Proliferation 38:347–355.

Matoušková E, Veselý P, Königová R. 1989. Modified method of *in vitro* cultivation of human keratinocytes suitable for grafting. Folia Biologica (Praha) 35:118–123.

Michel M, Török N, Godbout M-J, Lussier M, Gaudreau P, Royal A, Germain L. 1996. Keratin 19 as a biochemical marker of skin stem cells *in vivo* and *in vitro*: Keratin 19 expressing cells are differentially located in function of anatomic sites, and their number varies with donor age and culture stage. Journal of Cell Sciences 109:1017–1028.

Motlík J, Klíma J, Dvořánková B, Smetana K Jr. 2007. Porcine epidermal stem cells as a biomedical model for wound healing and normal/malignant epithelial cell propagation. Theriogenology 67:105–111.

Owens DM, Watt FM. 2003. Contribution of stem cells and differentiated cells to epidermal tumors. Nature Reviews Cancer 3:444–451.

Petersen OW, Lind Nielsen H, Gudjonsson T, Viladsen R, Rank F, Niebuhr E, Bissell MJ, Rønnov-Jenssen L. 2003. Epithelial to mesenchymal transition in human breast cancer can provide a nonmalignant stroma. American Journal of Pathology 162:391–402.

Plzák J, Smetana K Jr, Hrdličková E, Kodet R, Holíková Z, Liu F-T, Dvořánková B, Kaltner H, Betka J, Gabius H-J. 2001. Expression of galectin-3-reactive ligands in squamous cancer and normal epithelial cells as a marker of differentiation. International Journal of Oncology 19:59–64.

Plzák J, Betka J, Smetana K Jr, Chovanec M, Kaltner H, André S, Kodet R, Gabius H-J. 2004. Galectin-3: An emerging prognostic indicator in advanced head and neck carcinoma. European Journal of Cancer 40:2324–2330.

Purkrábková T, Smetana K Jr, Dvořánková B, Holíková Z, Böck C, Lensch M, André S, Pytlík R, Liu F-T, Klíma J, Smetana K, Motlík J, Gabius H-J. 2003. New aspects of galectin functionality in nuclei of cultured bone marrow stromal and epidermal cells: Biotinylated galectins as tool to detect specific binding sites. Biology of the Cell 95:535–545.

Raul U, Sawant S, Dange P, Kalraiya R, Ingle A, Vaidya M. 2004. Implications of cytokeratin 8/18 filament formation in stratified epithelial cells: Induction of transformed phenotype. International Journal of Cancer 111:662–668.

Reya T, Morrison SJ, Clake MF, Weissman IL. 2001. Stem cells, cancer and cancer stem cells. Nature 414:105–111.

Saiki RK, Scharf S, Faloona F, Mullis KB, Horn GT, Erlich HA, Arnheim N. 1985. Enzymatic amplification of β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science 230:1350–1354.

Sell S. 2004. Stem cell origin of cancer and differentiation therapy. Critical Reviews in Oncology Hematology 51: 1–28.

Smalley KS, Lioni M, Herlyn M. 2006. Life isn't flat: Taking cancer biology to the next dimension. In Vitro Cellular & Developmental Biology-Animal 42:242–247.

Smetana K Jr, Dvořánková B, Chovanec M, Bouček J, Klíma J, Motlík J, Lensch M, Kaltner H, André S, Gabius H-J. 2006. Nuclear presence of adhesion-/growth-regulatory galectins in normal/malignant cells of squamous epithelial origin. Histochemistry and Cell Biology 125:171–182.

Smith BD, Haffty BG. 1999. Molecular markers as prognostic factors for local recurrence and radioresistance in head and neck squamous cell carcinoma. Radiation Oncology Investigation 7:125–144.

Smith EM, Ritchie JM, Pawlita M, Rubenstein LM, Haugen TH, Turek LP, Hamšíková E. 2006. Human papilloma virus seropositivity and risks of head and neck cancer. International Journal of Cancer 120:825–832.

Szöke T, Kayser K, Baumhäkel J-D, Trojan I, Furak J, Tiszlavicz L, Horvath A, Szluka K, Gabius H-J, André S. 2005. Prognostic significance of adhesion/growth-regulatory lectins in lung cancer. Oncology 69:167–174.

Takkunem M, Grenman R, Hukkanen M, Korhonen M, Garcia de Herreros A, Virtanen I. 2006. Snail-dependent and -independent epithelial-mesenchymal transition in oral squamous carcinoma cells. Journal of Histochemistry & Cytochemistry 54:1263–1275.

Thiery J-P, Sleeman JP. 2006. Complex networks orchestrate epithelial-mesenchymal transitions. Nature Reviews Molecular Cell Biology 7:131–142.

Troy T-C, Turksen K. 2005. Commitment of embryonic stem cells to an epidermal cell fate and differentiation. Developmental Dynamics 232:293–300.

Tsai RYL, McKay RDG. 2002. A nucleolar mechanism controlling cell proliferation in stem cells and cancer cells. Genes Development 16:2991–3003.

Tumbar T, Guash G, Greco V, Blanpain C, Lowry WE, Rendl M, Fuchs E. 2004. Defining the epithelial stem cell niche in skin. Science 303:359 – 363.

van den Brule AJ, Pol R, Fransen-Daalmeijer N, Schouls LM, Meijer CJ, Snijders PJ. 2002. GP5+/6+ PCR followed by reverse line blot analysis enables rapid and high-throughput identification of human papillomavirus genotypes. Journal of Clinical Microbiology 40:779 – 787.

Villalobo A, Nogales-González A, Gabius H-J. 2006. A guide to signaling pathways connecting protein-glycan interaction with the emerging versatile effector functionality of mammalian lectins. Trends in Glycoscience and Glycotechnology 18:1 – 37.

Watt FM, Hoggan BL. 2000. Out of Eden: Stem cells and their niches. Science 287:1427 – 1430.

Weber F, Shen L, Fukino K, Patocs A, Mutter GL, Caldes T, Eng C. 2006. Total-genome analysis of BRCA1/2-related invasive carcinomas of the breast identifies tumor stroma as potential landscaper for neoplastic initiation. American Journal of Human Genetics 78:961 – 972.

Wu AM, Singh T, Wu JH, Lensch M, André S, Gabius H-J. 2006. Interaction profile of galectin-5 with free saccharides and mammalian glycoproteins: Probing its fine specificity and the effect of naturally clustered ligand presentation. Glycobiology 16:524 – 537.

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REVIEW

**Karel Smetana Jr · Barbora Dvořánková
Martin Chovanec · Jan Bouček · Jiří Klíma
Jan Motlík · Martin Lensch · Herbert Kaltner
Sabine André · Hans Joachim Gabius**

Nuclear presence of adhesion-/growth-regulatory galectins in normal/malignant cells of squamous epithelial origin

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Abstract Cellular activities in the regulation of growth or adhesion/migration involve protein (lectin)–carbohydrate recognition at the cell surface. Members of the galectin family of endogenous lectins additionally bind distinct intracellular ligands. These interactions with protein targets explain the relevance of their nuclear and cytoplasmic presence. Expression profiling for galectins and accessible binding sites is a histochemical approach to link localization with cellular growth properties. Non-cross-reactive antibodies for the homodimeric (proto-type) galectins-1, -2 and -7 and the chimera-type galectin-3 (Gal-3) as well as the biotinylated lectins were tested. This analysis was performed with the FaDu squamous carcinoma cell line and long-term cultured human and porcine epidermal cells as models for malignant and normal cells of squamous cell epithelial origin. A set of antibodies was added for phenotypic cell characterization. Strong nuclear and cytoplasmic signals of galectins and the differential reactivity of labeled galectins support the notion of their individual properties. The length of the period of culture was effective in modulating marker expression. Cytochemical expression profiling is a prerequisite for the selection of distinct proteins for targeted modulation of gene expression as a step toward functional analysis.

Keywords Galectin · Keratinocyte · Lectin · Nucleus · Nucleolus · Squamous cell · Stem cell

K. Smetana Jr (✉) · B. Dvořánková
Institute of Anatomy, 1st Faculty of Medicine, Charles University,
U nemocnice 3, Prague 2 128 00, Czech Republic
E-mail: ksmet@lf1.cuni.cz
Tel.: +420-2-24965873
Fax: +420-2-24965770

J. Klíma · J. Motlík · K. Smetana Jr · B. Dvořánková
Center of Cell Therapy and Tissue Repair,
2nd Faculty of Medicine, Charles University,
U nemocnice 3, Prague 2 128 00, Czech Republic

M. Chovanec · J. Bouček
Department of Otorhinolaryngology, Head and Neck Surgery,
Institute of Anatomy, 1st Faculty of Medicine, Charles University,
U nemocnice 3, Prague 2 128 00, Czech Republic

J. Bouček
Institute of Microbiology, Academy of Sciences of the Czech
Republic, Vídeňská 1083, Prague 4 140 08, Czech Republic

J. Klíma · J. Motlík
Institute of Animal Physiology and Genetics,
Academy of Sciences of the Czech Republic,
Rumburská 89, Liběchov 277 21, Czech Republic

M. Lensch · H. Kaltner · S. André · H. J. Gabius
Institute of Physiological Chemistry,
Faculty of Veterinary Medicine,
Ludwig-Maximilians-University, Veterinärstrasse 13,
Munich 80539, Germany

Background

Progress in cell biology is driven by the transition from descriptive observations to quantitative studies. Toward the ultimate aim of predicting cellular behavior based on the presence of certain cell components, expression profiling is essential and should be followed by thorough functional analysis. The more complex is the panel of compounds under study, the more demanding is the dimension of the given technical task. Viewed from the perspective of structural complexity, glycans clearly surpass nucleic acids and proteins and therefore qualify to be a high-density coding system (Gabius et al. 2004). The possibility to present a large number of different signals in a minimum of space on the cell surface and the dedication of a complex synthesis machinery for the production of the wide array of determinants favor the idea of this use (Reuter and Gabius 1999). Equally important, an implied information transfer from the glycan signals to cellular responses is enabled by the development of an intricate system of decoding devices, especially lectins (Gabius 1997; Kaltner and Stierstorfer 1998; Rüdiger and Gabius 2001; Solís et al. 2001). The remarkable specificity of lectin binding in terms of target

selection and the capacity for cross-linking are conducive to translate the sugar-encoded message into cellular activities such as growth regulation or endocytosis (Trombetta and Parodi 2002; Villalobo et al. 2005). Of central importance for this role are generally not the core constituents of glycan chains but the spatially accessible branch ends. Fittingly, they are subject to versatile modifications by the enzymatic addition of, for example, fucose, *N*-acetylgalactosamine (or its 4′-sulfated derivative) or sialic acid units to the β-galactoside core. The elaborate enzymatic systems for branch-end modifications, acting as switch on/off mechanisms for lectin binding, and the diversity of endogenous lectins strategically combine to form the complementary aspects for information transfer. Prominent in recent research on endogenous lectins, members of the galectin family are known to target precisely the branch-end structures.

Galectins: a link from sugar signals to cell responses

The currently characterized 14 proteins of this family of mammalian lectins are classified into monomeric/homodimeric proto-type lectins, tandem-repeat-type lectins in which a linker peptide covalently connects the two binding domains and the chimera-type Gal-3 in which the N-terminal domain harbors a short sequence stretch for phosphorylation and a collagenous stalk relevant for reversible aggregation (Gabius 1997, 2001; Lahm et al. 2004; Liu and Rabinovich 2005). The sugar specificity of the galectins extends beyond the β-galactoside core, and substitutions in its vicinity or even in the core region of the glycan chain significantly modulate ligand properties (Hirabayashi et al. 2002; Siebert et al. 2003; André et al. 2004a). Because the conformational flexibility of oligosaccharides is often limited to fluctuate between few, energetically privileged conformers, it is important to add that galectin-1 (Gal-1) selects such a distinct shape naturally adopted by the ligand so that a loss in entropy is minimized (Gabius et al. 2004). Moreover, spatial clustering of glycans has an important impact on galectin binding (André et al. 2004b; Wu et al. 2004).

Proto-type proteins such as human Gal-1 present binding sites for sugar at opposing sites of the homodimer and can even respond to this binding with a change of conformation (He et al. 2003; López-Lucendo et al. 2004). When targeting the same glycan on the cell surface, for example the pentasaccharide of ganglioside GM_1 on the surface of human SK-N-MC neuroblastoma cells, the different ways of ligand cross-linking by the galectin subfamilies can explain functional divergence. This was seen for galectins-1 and -3 (Kopitz et al. 2001; André et al. 2005). Of note, RT-PCR analysis and immunohistochemistry revealed that different cells can express more than one galectin type, a challenge for functional analysis of the network's players (Lahm et al. 2001; Kayser et al. 2003; Nagy et al. 2003). The emerging functional significance of galectins is documented in the clinical context by their description as prognostic markers and their activities, e.g. in tumor progression, clonal restriction of leukemic/activated T cell growth or dysfunction in cardiac hypertrophy (Rabinovich et al. 2002; Rappl et al. 2002; Nagy et al. 2003; Ozaki et al. 2004; Sharma et al. 2004; Sturm et al. 2004). As cellular effectors, the range of their activities can go beyond the interaction with surface glycan epitopes. In fact, galectins are not only present at the cell surface or in the extracellular matrix.

Immunohistochemical localization of galectins has started to provide evidence for galectin presence within the cell, nearly 20 years back (Gabius et al. 1986; Moutsatsos et al. 1986; Hubert et al. 1995). Targets for galectins at these sites are effectors of positive or negative growth regulation such as oncogenic H-ras, bcl-2, nucling or axin/β-catenin, constituents of small nuclear ribonucleoprotein particles and transcription factors such as TTF-1 (Lin et al. 2002; Liu et al. 2004; Paron et al. 2003; Liu 2004; Patterson et al. 2004; Rotblat et al. 2004; Shimura et al. 2005). Remarkably, a galectin can thus influence cell proliferation and apoptosis from the cell surface and also from intracellular sites. This apparent functional versatility and the distinct, often non-identical expression pattern for galectins, for example galectin-7 (Gal-7) present in squamous cell epithelia (Magnaldo et al. 1998) and galectins-1 and -3 in separate cell types (mesenchymal vs epithelial cells) in respiratory and digestive tracts (Kaltner et al. 2002), make galectins attractive study objects in the analysis of squamous cell epithelia.

Squamous cell epithelia and their galectins under physiological conditions and in cancer

The analysis of compartmentalization for distinct proteins in squamous cell epithelia using proteomics and genetic profiling is an active area of research. Only cells of the basal layer are able to proliferate. Similar to other types of tissues, this type of epithelium also contains stem cells (SCs) responsible for tissue renewal and regeneration. SCs are present in the basal layer of the interfollicular epidermis or at particular sites of the external root sheaths of hair follicles, the so-called bulge region (Watt 2002; Alonso and Fuchs 2003). One aim of profiling gene expression is to detect proteins, which are characteristic of these cell populations.

In our context, it is noteworthy that serial analysis of gene expression pinpointed Gal-1 as an abundantly expressed protein of mesenchymal stem cells (Silva et al. 2003), warranting immunocytochemical studies. At present, no specific markers of EpSCs are available but it is known that these cells express keratins (K)-15 and -19, which are characteristics of monolayer epithelia (Alonso and Fuchs 2003). Also, cells with EpSC features strongly express $\alpha_6\beta_4$- and β_1-integrins (Kaur and Li 2000;

Watt 2002). The notion that this issue has not yet been definitively resolved is supported by showing that cells with low level of β_1-integrin expression can also be classified as EpSCs (Ghazizadeh and Taichman 2005). Expression of p63, a transcription factor of the p53 family, in cell nuclei is also currently considered to be associated with stem cell properties of proliferative keratinocytes (Pellegrini et al. 2001; Koster et al. 2004). When monitoring initial steps of tumor development a supposedly crucial role of stem cells in cancer formation becomes apparent. They represent an almost ideal target for mutations which when accumulated might cause malignant transformation (Sell 2004). In this respect, galectins have been related to tumor progression via their association with tumor promoters or suppressors such as p53 (Lahm et al. 2004; Liu and Rabinovich 2005). This reasoning led us to examine galectin properties in squamous cell epithelia. In addition to immunohistochemical monitoring of galectin presence, labeled galectins were introduced as probes to visualize accessible binding sites. It is important to note that, despite sharing monosaccharide specificity with plant lectins, the ligand selections of tissue lectins can be notably different and that specific binding of intracellular protein sites is not mimicked by structurally non-homologous plant products.

The presence of several galectins in cells calls for stringent quality controls of the antibodies to exclude cross-reactivities. In such cases affinity depletion of the antiserum with the purified galectin(s) giving the undesired reaction is required, until quality controls by blotting and ELISA are satisfactory. Using specific antibody and labeled lectin, Gal-1 and Gal-1-reactive epitopes were detected in the cytoplasm of cells forming the layers of the interfollicular epidermis (Plzák et al. 2000). Gal-1-specific binding sites (Gal-1-BS) were furthermore expressed in the nuclei of keratinocytes located in the bulge region of that part of hair follicles which was also positive for K-19 (Klíma et al. 2005). No cells with these characteristics were observed in the interfollicular epidermis. Of note, cells in suspension prepared from interfollicular epidermis which originally were negative for both K-19 and Gal-1-BS acquired positivity during the process of initial attachment in vitro (Chovanec et al. 2004). In contrast, to attain expression, Gal-1-BS and p63 require a rather long period (minimally 1 week), if cells from hair follicles were kept in suspension (Purkrábková et al. 2003; Chovanec et al. 2004). In mice, the profile of presence of Gal-1 and Gal-3 is strictly regulated during the hair cycle (Wollina et al. 2000). The Gal-1-reactive sites in the nuclei of cultured cells can be spatially related with nuclear speckles harboring the splicing factor SC35 (Purkrábková et al. 2003), a result which is in line with a similar staining pattern in HeLa nuclei and Gal-1's ability to bind Gemin4 as a component of spliceosomes (Vyakarnam et al. 1998; Park et al. 2001). With respect to proliferation, cells prepared from hair follicles are able to maintain this activity for a longer period than cells prepared from interfollicular epidermis. In that case, binding of the endogenous lectin is shifted from the nucleus to the cytoplasm (Chovanec et al. 2004). After having given a detailed account of the cytochemical experiments with the proto-type Gal-1 it was pertinent to examine the corresponding parameters for the chimera-type Gal-3.

Gal-3 and Gal-3-BS were expressed suprabasally, predominantly in intercellular contacts of suprabasal cells of keratinized epithelia (Plzák et al. 2000, 2001). In comparison to other markers, the binding profile resembles that of desmosomal proteins (desmoplakins, desmogleins) when determined by simultaneous staining at the single-cell level (Plzák et al. 2001). Basal cells were negative or only weakly positive for these markers. In order to account for this difference, the hypothesis that regional differences in α2,6-sialylation, a modulatory signal for galectin reactivity, can occur was tested. Indeed, these basal cells were reactive with the *Sambucus nigra* agglutinin, which is specific for α2,6-linked sialyllactose in the terminal position of *N*-glycan chains. Enzymatic removal of the sialic acid moieties by sialidase treatment made the glycan accessible for Gal-3, illustrating the assumed impact of glycan substitution (Holíková et al. 2002). As was shown in normal cells, squamous carcinomas of head and neck can also present Gal-3-BS in the intercellular contacts. This feature proved to have prognostic relevance (Choufani et al. 1999; Plzák et al. 2004). In aggregate, the proto-type Gal-1 and chimera-type Gal-3 have different characteristics. The next issue to be resolved was to assess features of another proto-type galectin to define the level of cytochemically detectable overlap in this subfamily.

The study of the homodimeric Gal-7 (p53-induced gene-1) revealed that individual subfamily members should not be considered to be redundant. Gal-7 is typical for squamous epithelia, where it exerts a proapoptotic role after UV-B irradiation of keratinocytes (Magnaldo et al. 1998; Bernerd et al. 1999), whereas tumorigenesis in buccal squamous cell carcinoma is associated with upregulation of its expression (Chen et al. 2004). In the human DLD-1 colon cancer line, Gal-7 mRNA belongs to the 14 from 7,202 transcripts whose expression increased in p53-expressing cells (Polyak et al. 1997) and its overexpression led to growth inhibition of tumors inoculated to mice (Ueda et al. 2004). The complex nature of Gal-7 activities is further illustrated by the observations that differentiation is accompanied by Gal-7 expression in bladder squamous cell carcinoma, but chemically induced rat mammary carcinogenesis results in its overexpression, while it can play a dual role at different stages of thyroid malignancy (Lu et al. 1997; Østergaard et al. 1997; Rorive et al. 2002). In preliminary analysis, cells of squamous cell carcinomas of different origin appeared to express a rather low extent of Gal-7 compared to non-malignant epithelium, a finding that warrants thorough study (Magnaldo et al. 1998).

Having introduced three members of the galectin family and their currently studied characteristics in squamous epithelia, we next present results on monitoring these features together with markers assumedly relevant for stem cell properties and differentiation in the model system of the FaDu line of a squamous cell carcinoma with explicit consideration of the length of the culture period. In order to delineate common parameters when examining growth in vitro, we include analysis with long-term cultured porcine keratinocytes prepared from hair follicles.

Experimental details

Cells of the FaDu line were cultured in Minimum Essential Medium with Earle's salts supplemented with 2 mM L-glutamine, antibiotics (streptomycin and penicillin) and 10% fetal calf serum at 37°C and 5% CO_2. The cells were passaged regularly after 5 or 6 days. For the experiments, cells from the fourth subculture were harvested using a mixture of trypsin/EDTA (1:1), seeded on coverslips at a density of 1.5×10^4 cells/cm^2 and grown for 2 or 7 days, respectively, prior to cytochemical processing. Porcine dorsal skin was obtained from a local slaughterhouse, and hairs with the sheaths were obtained by microdissection. Thereafter, the prepared samples were placed in a tissue culture dish to allow keratinocytes to migrate from the sheaths to the plastic surface (Klíma et al. 2005). Subsequently, cells were harvested by trypsinization and passaged at a density of $6–7\times10^4$ cells/ml of high-glucose (4.5 g/l) Dulbecco's Minimal Essential Medium with 4 mM L-glutamine, 0.05 mM β-mercaptoethanol and antibiotics (streptomycin and penicillin) supplemented with 20% fetal calf serum. The cells were passaged after 3 or 4 days, and cells from the 31st passage were seeded on coverslips as described above.

For immuno- and galectin cytochemistry the cells were routinely fixed with 2% (w/v) paraformaldehyde in phosphate-buffered saline (pH 7.2) without or after previous permeabilization using Triton X-100. This detergent was selected based on recent observations of the sensitivity of nuclear Gal-1 presence to the type of reagent used for permeabilization (Vyakarnam et al. 1998). To saturate sites with protein-binding capacity the specimens were incubated with normal goat or swine serum or bovine serum albumin. Galectins-1, -2, -3 and -7 were detected by specific polyclonal antibodies tested for absence of cross-reactivity against other galectins (André et al. 1999; Nagy et al. 2003; Sturm et al. 2004), and Gal-3 was additionally visualized by the A1D6 monoclonal antibody directed against the N-terminal part of Gal-3 (Liu et al. 1996). Binding sites for galectins were visualized with biotinylated probes prepared under activity-preserving conditions (André et al. 1999) and checked for purity as well as the extent of labeling by two-dimensional gel electrophoresis (Purkrábková et al. 2003). In addition to the full-length protein the truncated version of Gal-3 was prepared by removing the collagenase-sensitive N-terminal part (Agrwal et al. 1993), rendering the probe suitable for colocalization of the lectin and its binding sites at the single-cell level.

To characterize the degree of differentiation, cell growth and the formation of adhesive contacts a panel of commercially available monoclonal antibodies (Table 1) was applied. Anti-keratin-8, -14 and -19 antibodies were used as differentiation markers to detect cell proliferation antibodies against Ki67 and the transcription factor p63, and the formation of intercellular contacts was analyzed with antibodies against desmoplakins and E-cadherin. Localization of β_1-integrin chain (CD29), the transferrin receptor (CD71), β-catenin and splicing factor SC35 were also included to monitor the cells' activation status and profile of nuclear speckles (Fu and Maniatis 1990) and as internal calibration standards for the spatial relationship to galectins-1 and -3, respectively. This marker panel thus includes nuclear, cytoplasmic and cell surface proteins and enabled us to monitor the functional status of the studied cells. ExtrAvidin-TRITC was employed as a second-step reagent for biotinylated galectins, while in immunocytochemistry FITC-labeled swine anti-mouse antibody or TRITC-labeled goat anti-mouse antibody was used. DNA in cell nuclei was labeled with DAPI. Staining of galectins and other antigens at the single-cell level followed a protocol described in detail previously (Froňková et al. 1999). Controls of specificity included assessment of antigen-dependent/antigen-independent staining with isotype-matched antibodies or by omission of first-step reagents as well as demonstration of sugar-dependent inhibition of galectin binding. Following this

Table 1 Marker selection for immunocytochemical analysis of FaDu cells

Detected antigen	Type of antibody	Supplier
Keratin-8	Mouse monoclonal	Dako, Brno, Czech Republic
Keratin-14	Mouse monoclonal	Sigma-Aldrich, Prague, Czech Republic
Keratin-19	Mouse monoclonal	Dako, Brno, Czech Republic
p63	Mouse monoclonal	Dako, Brno, Czech Republic
Ki67	Mouse monoclonal	Dako, Brno, Czech Republic
CD71	Mouse monoclonal	Dako, Brno, Czech Republic
E-cadherin	Mouse monoclonal	Dako, Brno, Czech Republic
Desmoplakins-1, −2	Mouse monoclonal	Progen, Heidelberg, Germany
CD29	Mouse monoclonal	Immunotech, Prague, Czech Republic
β-catenin	Rabbit polyclonal	Santa Cruz, Santa Cruz, USA
SC35	Mouse monoclonal	Sigma-Aldrich, Prague, Czech Republic

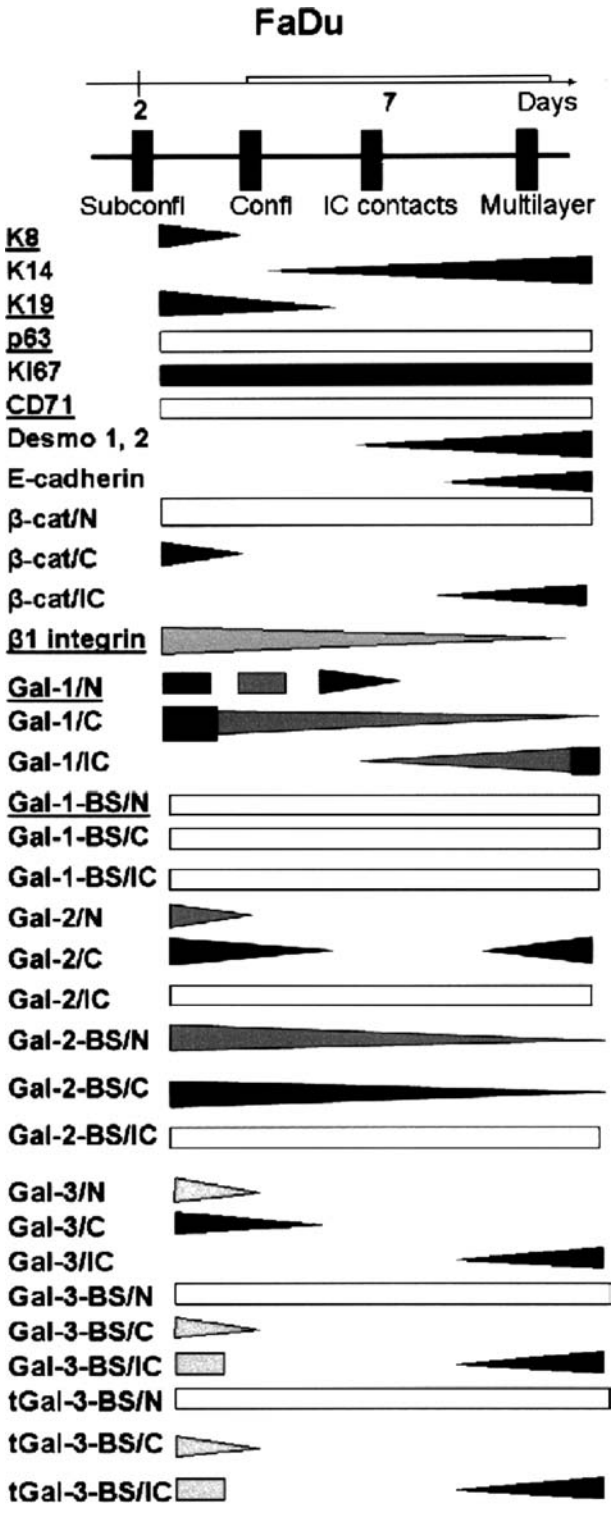

Fig. 1 Phenotypic characterization of cultured FaDu cells. Subconfluent (*Subconfl*) and confluent (*Confl*) cell populations as well as cells forming intercellular contacts (*IC*) and multilayers (*Multilayer*) were analyzed from the second to the seventh day of culture passage. Strong level of positivity is given in *black*, weak but distinct positivity in *gray* and lack of a specific signal in *white*. The trend for decreasing/increasing number of positive cells is indicated by the *wedge-like* part of the figure

process, specimens were exposed to Vectashield® mounting medium preventing UV bleaching of the samples and then analyzed with an Optiphot-2 fluorescence microscope equipped with filterblocks specific for FITC, TRITC or DAPI using the LUCIA computer-assisted image analysis system.

Immuno- and galectin cytochemical characterization of FaDu and porcine cells

Mapping of cell characteristics by cytochemical analysis was performed at two stages of cell culture. Subconfluent growth of cells with fibroblast-like morphology was observed 2 days after starting the cell culture. Cells of typically epithelial morphology forming a confluent monolayer with initial signs for multilayered growth were observed at the seventh day of culture. The formation of intercellular contacts is a prominent feature of a part of cells in monolayers as well as of cells in multilayered growth.

Phenotypic properties of cultured FaDu cells were significantly different between cell populations studied after the different culture periods. The results are summarized in a schematic presentation (Fig. 1). Keratins-8 and -19 were present in subconfluent monolayers of cells (Fig. 2a, a2), and reactivity for these intermediate filaments decreased when cells formed a confluent layer (Fig. 2b). In contrast, keratin-14 was detected in cells forming a confluent monolayer or a multilayer, in which positive cells established clusters of regular shape surrounded by cells giving no signal (Fig. 2e, f). Concerning nuclear markers, lack of p63 contrasted with a high level of expression of the Ki67 marker of proliferation, a result not influenced by the length of the culture period (Fig. 2g). Concerning markers on the cell surface, the cells were negative for expression of the transferrin receptor (CD71). Reactivity for desmoplakins-1 and -2 was observed in cells with intercellular contacts (Fig. 1). A similar pattern of expression, predominantly in cells of multilayered growth, concerned E-cadherin (Fig. 2c, d). β-Catenin was present in the cytoplasm of subconfluent cells and at sites of intercellular contacts of cells growing in multilayers. Interestingly, cells showed no evidence for nuclear accumulation of β-catenin. This pattern resembled the immunohistochemical detection of β-catenin in sections of oropharyngeal squamous cell cancers, intimating a role in cell adhesion rather than in signaling (Yu et al. 2005). The β_1-integrin subunit was present in subconfluent cells (not shown). Because β_1-integrin, β-catenin and a member of the cadherin family (LI-cadherin) are ligands for galectins-1, -2 and/or -3 (Furtak et al. 2001; Takamura et al. 2003; Sturm et al. 2004; Shimura et al. 2005), the next step was to analyze the expression of galectins and their accessible binding sites.

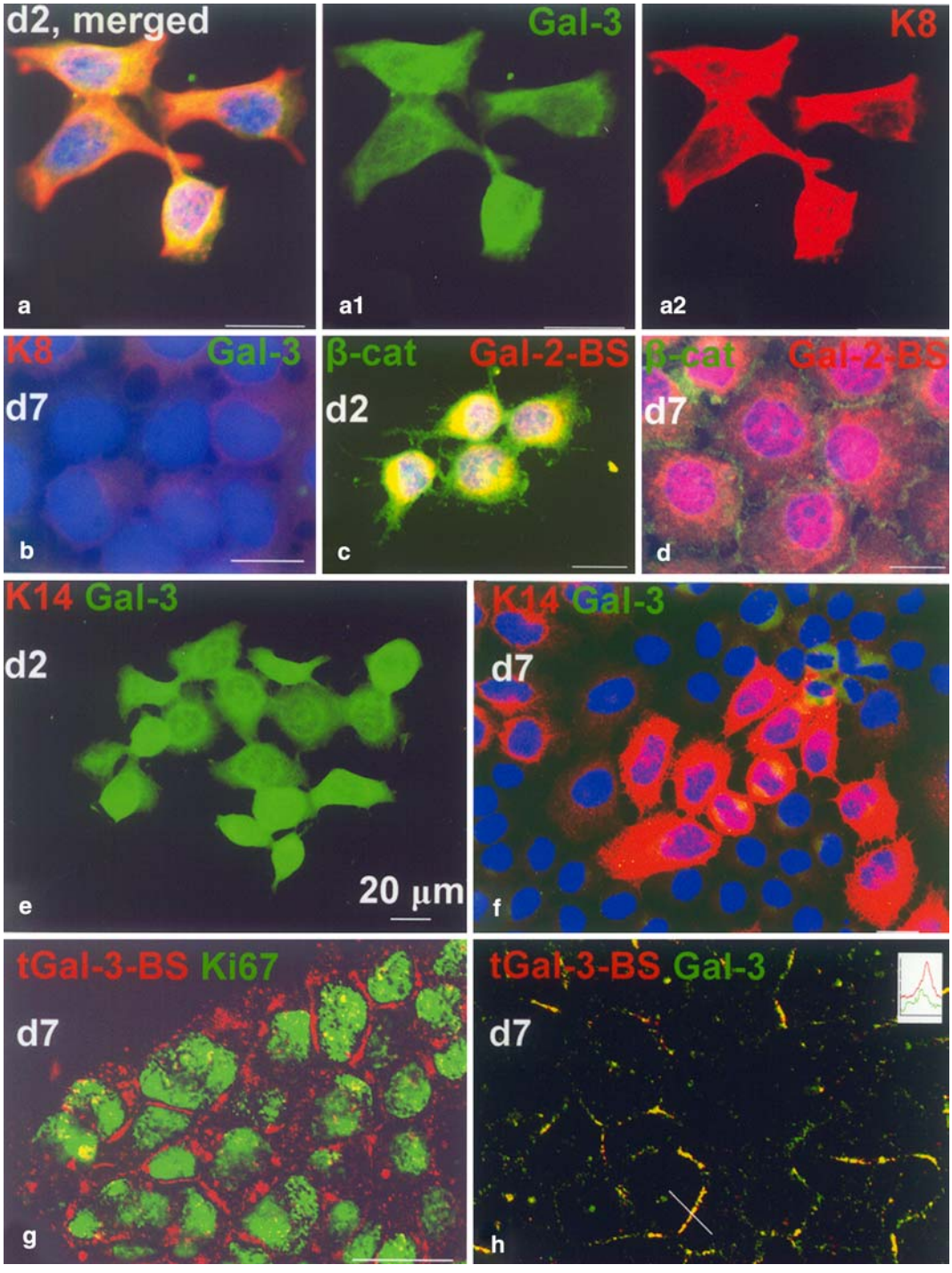

Fig. 2 Immunocytochemical detection of keratin-8 (K-8) (**a**, **a2**, **b**), β-catenin (β-cat) (**c**, **d**), keratin-14 (K-14) (**e**, **f**), Ki67 (**g**), galectin-3 (Gal-3) (**a**, **a1**, **b**, **e**, **f**, **h**), binding sites for galectin-2 (Gal-2-BS) (C, D) and binding sites for collagenase-treated (truncated) galectin-3 (tGal-3-BS) (**g**, **h**) in FaDu cells cultured for 2 (*d2*) or 7 (*d7*) days, respectively. Analysis of regional distribution of maximal intensity was used for the cases of detection of *tGal-3-BS* and Gal-3 (**h**). The profile of fluorescence intensity of intercellular contacts was measured and presented in the *inset* (**h**). Nuclei were counterstained with DAPI (**a**, **b**, **c**, **d**, **f**). Bar 20 μm

Gal-1 was localized in the nuclei with indication for localization in the nucleolus in subconfluent cells (Fig. 3a, b) and at the onset of confluent growth (Fig. 3d, e). Previous work has used fibrillarin and nucleophosmin for this assignment (Purkrábková et al. 2003). This reactivity was negatively correlated to further growth, and a part of the cell population in confluent growth forming extensive intercellular contacts and multilayers lacked this signal (see encircled cell in Fig. 3d). Extranucleolar positivity showed at least spatial vicinity to SC35-positive speckles (Fig. 4a–a3). Aside from nuclear presence, Gal-1 was also detected in

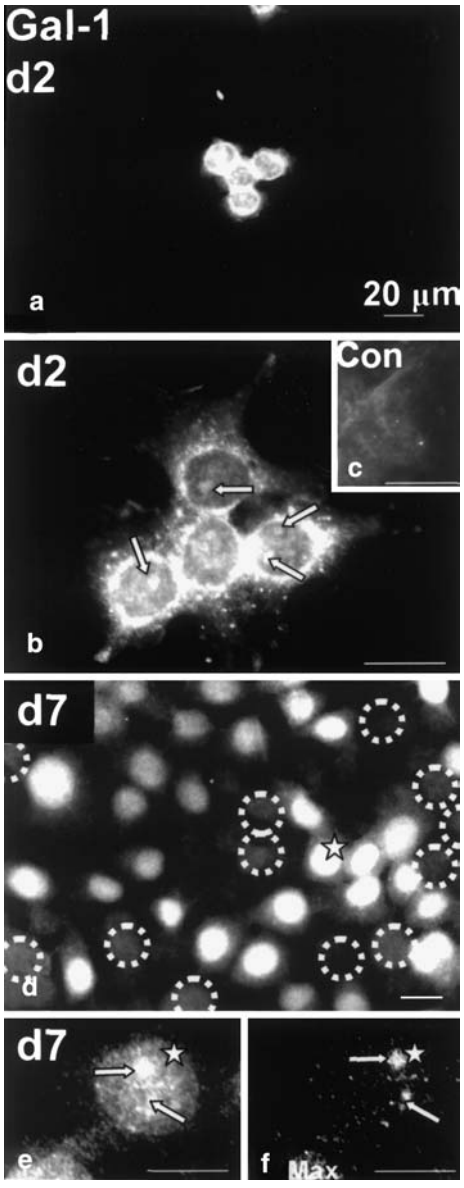

Fig. 3 Immunocytochemical detection of galectin-1 (Gal-1) in FaDu cells cultured for 2 days (*d2*) (**a**, **b**) with a control using an antiserum against a C-type lectin of macrophages to exclude antigen-independent staining (**c**) and for 7 days (*d7*) (**d**), respectively. High level of magnification of the cell from panel **c** marked with an *asterisk* after short-term exposure (**e**) and after computation of the regional distribution of maximal staining intensity by computer-assisted analysis (E_{Max}). Cells shown in panel **d** were overexposed to demonstrate the mosaic-like pattern of cells with confluent growth. *Arrows* mark the strong nucleolar signal (**a**, **d**, **d1**). *White circles* denote cells which lack expression of Gal-1 after a culture period of 7 days. Bar 20 µm

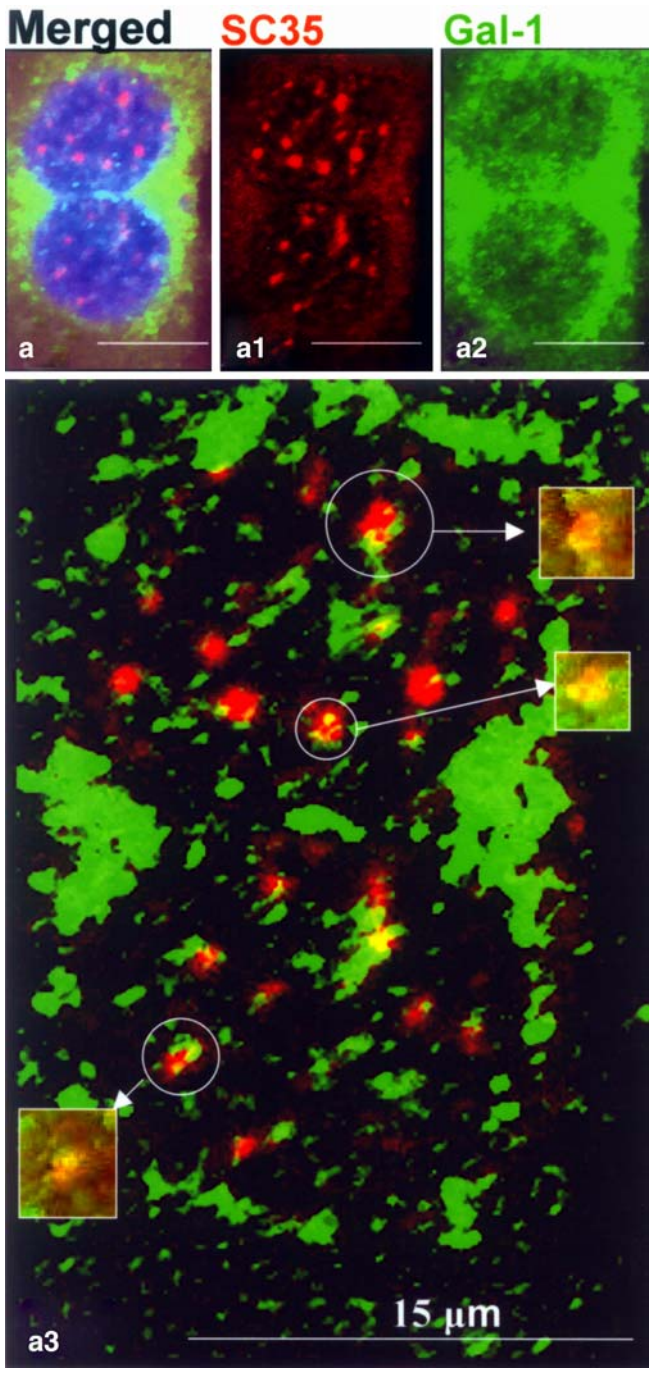

Fig. 4 Immunocytochemical detection of the splicing factor SC35 (**a**, **a1**, **a3**) and Gal-1 (Gal-1) (**a**, **a2**, **a3**). The regional distribution of maximal intensity is shown in **a3**, where fluorescence signals of the *encircled parts* were processed with the image analyzer module for three-dimensional modeling (*quadrangular insets*). Nuclei were counterstained with DAPI (**a**). The *weak red* cytoplasmic signal was due to cell autofluorescence. Bar 15 µm

the cytoplasm of subconfluent cells, and the number of positive cells continuously decreased when cells established multilayers (Fig. 3c). Evidently, the immunocytochemical findings on Gal-1 are influenced by the growth pattern of these cells in vitro. This result deserves consideration when discussing the heterogeneity of galectin expression in tumors. Despite positive controls documenting its reactivity, labeled Gal-1 failed to bind to any cells in a specific manner (Fig. 1).

Next, we turned to monitor the expression of Gal-2 (Gal-2), a proto-type galectin with close structural similarity to Gal-1 but distinct binding and activity

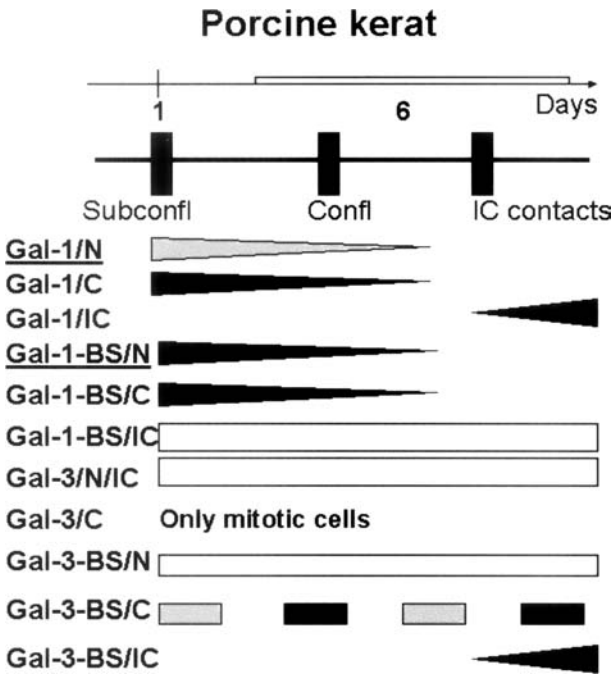

Fig. 5 Phenotypic characterization of long-term passaged porcine keratinocytes (*Porcine kerat*) derived from hair follicle cells. Subconfluent (*Subconfl*) and confluent (*Confl*) cell populations as well as cells forming intercellular contacts (*IC*) were analyzed from the first to the sixth day of culture passage. Strong level of positivity is given in *black*, weak but distinct positivity in *gray* and lack of a specific signal in *white*. The trend for decreasing/increasing number of positive cells is indicated by the *wedge-like* part of the figure

properties, experimentally evaluated on activated T cells (Sturm et al. 2004). Intracellular ligands for Gal-2 comprise lymphotoxin-α and α- and β-tubulins (Ozaki et al. 2004). The expression of this proto-type galectin in the nuclei of subconfluent cells was intense in nucleoli (Fig. 1). In subconfluent or early confluent cells and in cells forming multilayers Gal-2 is present in the cytoplasm and also, in contrast to Gal-1, in intercellular contacts (Fig. 1). A further difference to Gal-1 became apparent when reactivity to biotinylated Gal-2 was detected in the nuclei and cytoplasm: the intensity of staining of Gal-2-reactive epitopes decreased with the length of the culture period (Fig. 2c, d).

Gal-3, the only chimera-type family member, is a potent regulator of cell proliferation/apoptosis and also of adhesion/migration. The general concept that galectins can form networks of expression is supported by the detection of its presence in the nuclei and cytoplasm in subconfluent cells (Fig. 2e). Intercellular contacts were positive in multilayers, as likewise seen for Gal-1 (Fig. 2h). Binding of biotinylated Gal-3 was seen only in the cytoplasm of cells in the subconfluent stage whereas nuclei were negative. The intercellular contacts of cells in the subconfluent stage were rarely stained, while multilayers showed strong intensity at these sites (Fig. 1). A similar staining pattern was observed when the collagenase-sensitive N-terminal domain had enzymatically been removed. We conclude that the reactivity can solely be attributed to the activity of the carbohydrate recognition domain. Experiments to localize a third proto-type galectin, i.e. Gal-7, were invariantly negative, corroborating the lack of presence of Gal-7 in the TR146, A431 and SCC13 squamous cell carcinoma lines and in keratinocytes (K-14) after SV40-dependent transformation (Madsen et al. 1995; Magnaldo et al. 1998).

When monitoring the normal cells, subconfluent cell populations were observed after 1 day of culture and confluence was reached at about day 6. In contrast to the FaDu cells these cultures contained populations usually being round with epitheloid-like morphology devoid of a fibroblastoid pattern. As expected for non-malignant cells, contact inhibition was active. Due to the species difference we restricted the analysis to monitoring of galectin-dependent parameters, which are presented in Fig. 5. In detail, Gal-1 was expressed in subconfluent to early confluent cell populations in the nuclei including prominent presence in the nucleoli (Fig. 6a), in the cytoplasm with positivity around the cell nucleus and in the intercellular contacts (Fig. 6b). Compared to Gal-1, the chimera-type galectin was only detected in mitotic cells. Reactivity for Gal-3-BS was seen in the cytoplasm in few cells only, where reactivity was observed surrounding the nuclei (Fig. 6c). Strong staining intensity was detected in intercellular contacts of cells at the periphery of large colonies (Fig. 6c). The cells in the center of a colony were almost negative (Fig. 6c). Subconfluent cells in the vicinity of the edge of a colony presented a strong perinuclear signal (Fig. 6d). Using double labeling, where also DNA in nuclei was labeled, lack of Gal-3-reactivity for the majority of cells in the vicinity of these large colonies was measured (Fig. 6c, c1).

Galectin-related parameters, phenotypic characterization and differentiation

One of the main findings of our experiments is that subconfluent FaDu cells showed positive staining reactions applying antibodies against marker molecules upregulated in EpSCs, and the presence of these markers is decreased when cell populations reach confluence or form multilayers. For example, keratin-8 is expressed in embryoid bodies in prospective EpSCs (Troy and Turksen 2005) and considering malignancy, this type of intermediate filament is also expressed in poorly differentiated head and neck squamous cell carcinomas (Gires et al. 2004). By transfection of non-malignant cells with keratin-8-specific cDNA, a malignant phenotype is established (Casanova et al. 2004; Raul et al. 2004). Moreover, keratin-19 is considered as a marker of EpSC (see above). Its expression in FaDu resembles keratin-8 presence. Our previous study with keratin-19-negative interfollicular human keratinocytes had shown a similar transient expression of this intermediate filament induced by attachment of cells from

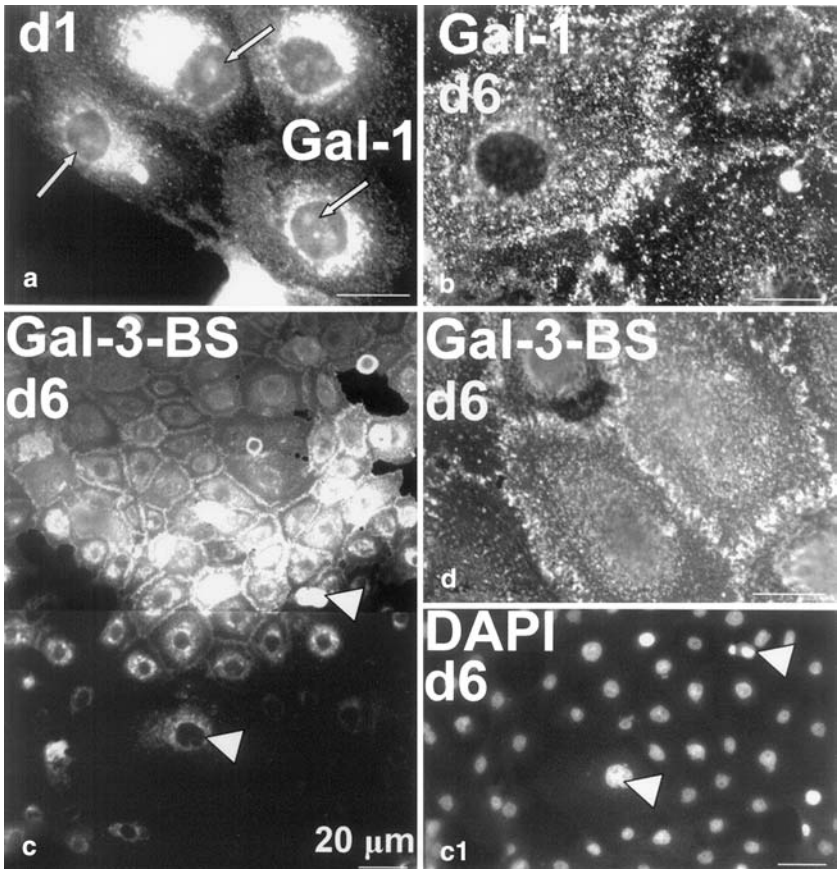

Fig. 6 Immunocytochemical detection of Gal-1 (**a**, **b**), Gal-3-BS (**c**, **d**) and visualization of nuclei counterstained with DAPI (**c1**) in porcine keratinocytes (31st passage) maintained in culture for 1 day (*d1*) and 6 days (*d6*), respectively. *Arrows* (**a**) and *arrowheads* (**c**, **c1**) show the position of nucleoli. Bar 20 µm

suspension culture (Chovanec et al. 2004). Keratin-14 belongs to the markers of basal cells (Freedberg et al. 2001). FaDu cells positive for keratin-14 were seen in confluent colonies. Expression of p63, which is present in the nuclei of EpSCs (Pellegrini et al. 2001; Koster et al. 2004), was absent in FaDu cells. The positivity for Ki67 reflected the proliferative activity of the tumor cells. Additionally, lack of expression of the transferrin receptor (CD71) may indicate a low differentiation level and extensive proliferative activity, because the proliferating SC pool is usually negative for this marker (Webb et al. 2004). Desmosomal proteins desmoplakin-1 and -2 as well as E-cadherin are associated with intercellular contacts in confluent monolayers of cells. In parallel, β-catenin is expressed in the cytoplasm of subconfluent cell populations and also present in intercellular contacts in confluent monolayers of cells. Its nuclear expression in cells of hair follicles is interpreted as an indication for growth activity originating from the EpSCs-derived cell lineage (Rindapaa et al. 2001; Lyakhovitsky et al. 2004). Regarding the lectin aspect our results add to the notion that galectins harbor more activities than expected from a typical lectin. Gal-1 presence was as abundant in nuclei as it was in intercellular contacts. It is also noteworthy that two proto-type galectins, namely Gal-1 and Gal-2, have different binding profiles.

Concluding remarks

Having herewith taken a further step toward delineating the way the complex galectin network may operate, it is of consequence to pursue this route by analysis of tandem-repeat-type galectins such as galectins-4, -8 or -9. Galectin-4, for example, has a nuclear localization in breast and colon carcinoma cells and interacts with the apoptosis-associated protein nucling as does Gal-3, whereas nuclear localization was found to level-off in malignancy for galectin-8 (Danguy et al. 2001; Nagy et al. 2003; Huflejt and Leffler 2004; Liu et al. 2004). With focus on its carbohydrate-binding activity to sulfatides with long-chain hydroxylated acyl chains (C_{24}) RNA interference taught the lesson that this mode of interaction is involved in clustering of lipid rafts for apical delivery in human HT-29 colon carcinoma cells (Delacour et al. 2005). At present, the cyto- and histochemical galectin fingerprinting is far from being complete, the next challenge to be addressed.

Equally important, ectopic expression of Gal-7 in squamous cell carcinomas is a way to link this protein, if possible, to develop an innovative therapeutic strategy. Of note in this respect, the nuclear activities implied by respective localization deserve special attention. It is thus a biochemical challenge to structurally define the

sites which are in contact with protein ligands. If separate from the carbohydrate-binding site, the question on interplay between the two sites will have to be resolved. Looking beyond galectins, experiments with neoglycoproteins document the presence of sugar receptors on keratinocytes, cells of the SCL-1 squamous carcinoma cell line and sections of squamous cell carcinomas with specificities to galactose-6-phosphate, α-L-rhamnose or α-L-fucose, broadening the scope of analysis of lectins in this cell system (Gabius et al. 1990; Cerdan et al. 1991; Condaminet et al. 1997; Minwalla et al. 2001; Szolnoky et al. 2001). Their biochemical characterization will add to the panel of markers, because activities were found to be UV-B inducible, regulatable by cytokines or partaking in melanosome transfer. In aggregate, the combination of cyto- and histochemical with biochemical analysis as well as the design of lectin and cell variants for functional investigations holds the promise to clarify the role of individual lectins as extracellular, cytoplasmic and nuclear effectors.

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References

Agrwal N, Sun Q, Wang SY, Wang JL (1993) Carbohydrate-binding protein 35. I. Properties of the recombinant polypeptide and the individuality of the domains. J Biol Chem 268:14932–14939

Alonso L, Fuchs E (2003) Stem cells of the skin epithelium. Proc Natl Acad Sci USA 100:11830–11835

André S, Kojima S, Yamazaki N, Fink C, Kaltner H, Kayser K, Gabius H-J (1999) Galectins-1 and -3 and their ligands in tumor biology. J Cancer Res Clin Oncol 125:461–474

André S, Unverzagt C, Kojima S, Frank M, Seifert J, Fink C, Kayser K, von der Lieth C-W, Gabius H-J (2004a) Determination of modulation of ligand properties of synthetic complex-type biantennary *N*-glycans by introduction of bisecting GlcNAc *in silico*, *in vitro* and *in vivo*. Eur J Biochem 271:118–134

André S, Kaltner H, Furuike T, Nishimura S-I, Gabius H-J (2004b) Persubstituted cyclodextrin-based glycoclusters as inhibitors of protein–carbohydrate recognition using purified plant and mammalian lectins and wild-type and lectin-gene-transfected tumor cells as targets. Bioconjugate Chem 15:87–98

André S, Kaltner H, Lensch M, Russwurm R, Siebert H-C, Fallsehr C, Tajkhorshid E, Heck AJR, von Knebel-Doeberitz M, Gabius H-J, Kopitz J (2005) Determination of structural and functional overlap/divergence of five proto-type galectins by analysis of the growth-regulatory interaction with ganglioside GM_1 *in silico* and *in vitro* on human neuroblastoma cells. Int J Cancer 114:46–57

Bernerd F, Sarasin A, Magnaldo T (1999) Galectin-7 overexpression is associated with the apoptotic process in UVB-induced sunburn keratinocytes. Proc Natl Acad Sci USA 96:11329–11334

Casanova ML, Bravo A, Martinez-Palacio J, Fernandez-Acenero MJ, Villanueva C, Larcher F, Conti CJ, Jorcano JL (2004) Epidermal abnormalities and increased malignancy of skin tumors in human epidermal keratin 8-expressing transgenic mice. FASEB J 18:1556–1558

Cerdan D, Grillon C, Monsigny M, Redziniak G, Kieda C (1991) Human keratinocyte membrane lectins: characterization and modulation of their expression by cytokines. Biol Cell 73:35–42

Chen J, He QY, Yuen APW, Chin JF (2004) Proteomics of buccal squamous cell carcinoma: the involvement of multiple pathways in tumorigenesis. Proteomics 4:2465–2475

Choufani G, Nagy N, Saussez S, Marchant H, Bisschop P, Burchert M, Danguy A, Louryan S, Salmon I, Gabius H-J, Kiss R, Hassid S (1999) The levels of expression of galectin-1, galectin-3, and the Thomsen–Friedenreich antigen and their binding sites decrease as clinical aggressiveness increases in head and neck cancers. Cancer 86:2353–2363

Chovanec M, Smetana K Jr, Dvořánková B, Plzáková Z, André S, Gabius H-J (2004) Decrease of nuclear reactivity to growth-regulatory galectin-1 in senescent human keratinocytes and detection of non-uniform staining profile alterations upon prolonged culture for galectins-1 and -3. Anat Histol Embryol 33:348–354

Condaminet B, Redziniak G, Monsigny M, Kieda C (1997) Ultraviolet rays induced expression of lectins on the surface of a squamous carcinoma keratinocyte cell line. Exp Cell Res 232:216–224

Cooper DNW (2002) Galectinomics: finding themes in complexity. Biochim Biophys Acta 1572:209–231

Craig SS, Krishnaswamy P, Irani AM, Kepley CL, Liu F-T, Schwartz LB (1995) Immunoelectron microscopic localization of galectin-3, an IgE binding protein, in human mast cells and basophils. Anat Rec 242:211–219

Danguy A, Rorive S, Decaestecker C, Bronckart Y, Kaltner H, Hadari YR, Goren R, Zick Y, Petein M, Salmon I, Gabius H-J, Kiss R (2001) Immunohistochemical profile of galectin-8 expression in benign and malignant tumors of epithelial, mesenchymatous and adipous origins, and of the nervous system. Histol Histopathol 16:861–868

Delacour D, Gouyer V, Zanetta J-P, Drobecq H, Leteurtre E, Grard G, Moreau-Hannedouche O, Maes E, Pons A, André S, Le Bivic A, Gabius H-J, Manninen A, Simons K, Huet G (2005) Galectin-4 and sulfatides in apical membrane trafficking in enterocyte-like cells. J Cell Biol 169:491–501

Freedberg IM, Tomic-Canic M, Komine M, Blumenberg M (2001) Keratins and the keratinocyte activation cycle. J Invest Dermatol 116:633–640

Froňková V, Holíková Z, Liu F-T, Homolka J, Rijken DC, André S, Bovin NV, Smetana K Jr, Gabius H-J (1999) Simultaneous detection of endogenous lectins and their binding capacity at the single cell-level—a technical note. Folia Biol 45:157–162

Fu X-D, Maniatis T (1990) Factor required for mammalian spliceosome assembly is localized in discrete regions in the cell nucleus. Nature 343:437–441

Furtak V, Hatcher F, Ochieng J (2001) Galectin-3 mediates the endocytosis of β_1-integrins by breast carcinoma cells. Biochem Biophys Res Commun 289:845–850

Gabius H-J (1997) Animal lectins. Eur J Biochem 243:543–576

Gabius H-J (2001) Glycohistochemistry: the why and how of detection and localization of endogenous lectins. Anat Histol Embryol 30:3–31

Gabius H-J, Brehler R, Schauer A, Cramer F (1986) Localization of endogenous lectins in normal human breast, benign breast lesions and mammary carcinomas. Virchows Arch B [Cell Pathol] 52:107–115

Gabius H-J, Heil MS, Berger H (1990) Glycohistochemistry of endogenous lectins in cutaneous cancer. Anticancer Res 10:1627–1631

Gabius H-J, Siebert H-C, André S, Jiménez-Barbero J, Rüdiger H (2004) Chemical biology of the sugar code. ChemBioChem 5:740–764

Ghazizadeh S, Taichman LB (2005) Organization of stem cells and their progeny. J Invest Dermatol 124:367–372

Gires O, Münz M, Schaffrik M, Kieu C, Rauch J, Ahlemann M, Eberle D, Mack B, Wollenberg B, Lang S, Hofmann T, Hammerschmidt W, Zeidler R (2004) Profile identification of disease-associated humoral antigens using AMIDA, a novel proteomics-based technology. Cell Mol Life Sci 61:1198–1207

He L, André S, Siebert H-C, Helmholz H, Niemeyer B, Gabius H-J (2003) Detection of ligand- and solvent-induced shape alterations of cell-growth-regulating human galectin-1 in solution by small angle neutron and X-ray scattering. Biophys J 85:511–524

Hirabayashi J, Hashidate T, Arata Y, Nishi N, Nakamura T, Hirashima M, Urashima T, Oka T, Futai M, Müller WEG, Yagi F, Kasai K-i (2002) Oligosaccharide specificity of galectins: a search by frontal affinity chromatography. Biochim Biophys Acta 1572:232–254

Holíková Z, Hrdličková-Cela E, Plzák J, Smetana K Jr, Betka J, Dvořánková B, Esner M, Wasano K, André S, Kaltner H, Motlík J, Hercogová J, Kodet R, Gabius H-J (2002) Defining the glycophenotype of squamous epithelia by plant and mammalian lectins. Differentiation-dependent expression of α2,6- and α2,3-linked *N*-acetylneuraminic acid in squamous epithelia and carcinomas and its differential effect on binding of the endogenous lectins galectins-1 and -3. APMIS 110:845–856

Hubert M, Wang SY, Wang JL, Seve AP, Hubert J (1995) Intranuclear distribution of galectin-3 in mouse 3T3 fibroblasts: comparative analyses by immunofluorescence and immunoelectron microscopy. Exp Cell Res 220:397–406

Huflejt ME, Leffler H (2004) Galectin-4 in normal tissues and cancer. Glycoconj J 20:247–255

Kaltner H, Stierstorfer B (1998) Animal lectins as cell adhesion molecules. Acta Anat (Basel) 161:162–179

Kaltner H, Seyrek K, Heck A, Sinowatz F, Gabius H-J (2002) Galectin-1 and galectin-3 in fetal developmental of bovine respiratory and digestive tracts. Cell Tissue Res 307:35–46

Kaur P, Li A (2000) Adhesive properties of human epidermal cells: an analysis of keratinocyte stem cells. J Invest Dermatol 114:413–420

Kayser K, Hoeft D, Hufnagl P, Caselitz J, Zick Y, André S, Kaltner H, Gabius H-J (2003) Combined analysis of tumor growth pattern and expression of endogenous lectins as a prognostic tool in primary testicular cancer and its lung metastases. Histol Histopathol 18:771–779

Klíma J, Smetana K Jr, Motlík J, Plzáková Z, Liu F-T, Štork J, Kaltner H, Chovanec M, Dvořánková B, André S, Gabius H-J (2005) Comparative phenotypic characterization of keratinocytes originating from hair follicles. J Mol Histol 36:89–96

Kopitz J, von Reitzenstein C, André S, Kaltner H, Uhl J, Ehemann V, Cantz M, Gabius H-J (2001) Negative regulation of neuroblastoma cell growth by carbohydrate-dependent surface binding of galectin-1 and functional divergence from galectin-3. J Biol Chem 276:35917–35923

Koster M, Kim S, Mills AA, DeMayo FJ, Roop DR (2004) *p63* is the molecular switch for initiation of an epithelial stratification program. Genes Dev 18:126–131

Lahm H, André S, Hoeflich A, Fischer JR, Sordat B, Kaltner H, Wolf E, Gabius H-J (2001) Comprehensive galectin fingerprinting in a panel of 61 human tumor cell lines by RT-PCR and its implications for diagnostic and therapeutic procedures. J Cancer Res Clin Oncol 127:375–386

Lahm H, André S, Hoeflich A, Kaltner H, Siebert H-C, Sordat B, von der Lieth C-W, Wolf E, Gabius H-J (2004) Tumor galectinology: insights into the complex network of a family of endogenous lectins. Glycoconj J 20:227–238

Lin H-M, Pestell RG, Raz A, Choi-Kim H-R (2002) Galectin-3 enhances cyclin D_1 promoter activity through SP1 and a cAMP-responsive element in human breast epithelial cells. Oncogene 21:8001–8010

Liu F-T (2004) Double identity: galectins may not function as lectins inside the cell. Trends Glycosci Glycotechnol 16:255–264

Liu F-T, Rabinovich GA (2005) Galectins as modulators of tumor progression. Nat Rev Cancer 5:29–41

Liu F-T, Hsu DK, Zuberi RI, Hill PN, Shenhav A, Kuwabara I, Chen SS (1996) Modulation of functional properties of galectin-3 by monoclonal antibodies binding to the non-lectin domains. Biochemistry 35:6073–6079

Liu L, Sakai T, Sano N, Fukui K (2004) Nucling mediates apoptosis by inhibiting expression of galectin-3 through interference with nuclear factor κB signalling. Biochem J 380:31–41

López-Lucendo MF, Solís D, André S, Hirabayashi J, Kasai K-i, Kaltner H, Gabius H-J, Romero A (2004) Growth-regulatory human galectin-1: crystallographic characterisation of the structural changes induced by single-site mutations and their impact on the thermodynamics of ligand binding. J Mol Biol 343:957–970

Lu J, Pei H, Kaeck M, Thompson HJ (1997) Gene expression changes associated with chemically induced rat mammary carcinogenesis. Mol Carcinog 20:204–215

Lyakhovitsky A, Barzilai A, Fogel M, Trau H, Huszar M (2004) Expression of E-cadherin and β-catenin in cutaneous squamous cell carcinoma and its precursors. Am J Dermatopathol 26:372–378

Madsen P, Rasmussen HH, Flint T, Gromov P, Kruse TA, Honore B, Vorum H, Celis JE (1995) Cloning, expression, and chromosome mapping of human galectin-7. J Biol Chem 270:5823–5829

Magnaldo T, Fowlis D, Darmon M (1998) Galectin-7, a marker of all types of stratified epithelia. Differentiation 63:159–168

Minwalla L, Zhao Y, Cornelius J, Babcock GF, Wickett RR, Le Poole IC, Boissy RE (2001) Inhibition of melanosome transfer from melanocytes to keratinocytes by lectins and neoglycoproteins in an *in vitro* model system. Pigment Cell Res 14:185–194

Moutsatsos IK, Davis JM, Wang JL (1986) Endogenous lectins from cultured cells: subcellular localization of carbohydrate-binding protein 35 in 3T3 fibroblasts. J Cell Biol 102:477–483

Nagy N, Legendre H, Engels O, André S, Kaltner H, Wasano K, Zick Y, Pector JC, Decaestecker C, Gabius H-J, Salmon I, Kiss R (2003) Refined prognostic evaluation in colon carcinoma using immunohistochemical galectin fingerprinting. Cancer 97:1849–1858

Østergaard M, Rasmussen HH, Nielsen HV, Vorum H, Orntoft TF, Wolf H, Celis JE (1997) Proteome profiling of bladder squamous cell carcinomas: identification of markers that define their degree of differentiation. Cancer Res 57:4111–4117

Ozaki K, Inoue K, Sato H, Iida A, Ohnishi Y, Sekine A, Sato H, Odashira K, Nobuyoshi M, Hori M, Nakamura Y, Tanaka T (2004) Functional variation in LGALS2 confers risk of myocardial infarction and regulates lymphotoxin-α secretion *in vitro*. Nature 429:72–75

Park JW, Voss PG, Grabski S, Wang JL, Patterson RJ (2001) Association of galectin-1 and galectin-3 with Gemin4 in complex containing SMN protein. Nucleic Acids Res 27:3595–3602

Paron I, Scaloni A, Pines A, Bachi A, Liu F-T, Puppin C, Pandolfi M, Ledda L, Di Loreto C, Damante G, Tell G (2003) Nuclear localization of galectin-3 in transformed thyroid cells: a role in transcriptional regulation. Biochem Biophys Res Commun 302:545–553

Patterson RJ, Wang W, Wang JL (2004) Understanding the biochemical activities of galectin-1 and galectin-3 in the nucleus. Glycoconj J 19:499–506

Pellegrini G, Dellambra E, Goliosano O, Martinelli E, Fantozi I, Bondanza S, Ponzin D, McKeon F, De Luca M (2001) p63 identifies keratinocyte stem cells. Proc Natl Acad Sci USA 98:3156–3161

Plzák J, Smetana K Jr, Betka J, Kodet R, Kaltner H, Gabius H-J (2000) Endogenous lectins (galectins-1 and -3) as probes to detect differentiation-dependent alterations in human squamous cell carcinomas of oropharynx and larynx. Int J Mol Med 5:369–372

Plzák J, Smetana K Jr, Hrdličková E, Kodet R, Holíková Z, Liu F-T, Dvořánková B, Kaltner H, Betka J, Gabius H-J (2001) Expression of galectin-3-reactive ligands in squamous cancer and normal epithelial cells as a marker of differentiation. Int J Oncol 19:59–64

Plzák J, Betka J, Smetana K Jr, Chovanec M, Kaltner H, André S, Kodet R, Gabius H-J (2004) Galectin-3: an emerging prognostic indicator in advanced head and neck carcinoma. Eur J Cancer 40:2324–2330

Polyak K, Xia Y, Zweier JL, Kinzler KW, Vogelstein B (1997) A model for p53-induced apoptosis. Nature 389:300–305

Purkrábková T, Smetana K Jr, Dvořánková B, Holíková Z, Böck C, Lensch M, André S, Pytlík R, Liu F-T, Klíma J, Smetana K Jr, Motlík J, Gabius H-J (2003) New aspects of galectin functionality in nuclei of cultured bone marrow of stromal and epidermal cells: biotinylated galectins as tool to detect specific binding sites. Biol Cell 95:535–545

Rabinovich GA, Rubinstein N, Toscano MA (2002) Role of galectins in inflammatory and immunomodulatory processes. Biochim Biophys Acta 1572:274–284

Rappl G, Abken H, Muche JM, Sterry W, Tilgen W, André S, Kaltner H, Ugurel S, Gabius H-J, Reinhold U (2002) $CD4^+CD7^-$ leukemic T cells from patients with Sézary syndrome are protected from galectin-1-triggered T cell death. Leukemia 16:840–845

Raul U, Sawant S, Dange P, Kalraiya R, Ingle A, Vaidya M (2004) Implications of cytokeratin 8/18 filament formation in stratified epithelial cells: induction of transformed phenotype. Int J Cancer 111:662–668

Reuter G, Gabius H-J (1999) Eukaryotic glycosylation: whim of nature or multipurpose tool? Cell Mol Life Sci 55:368–422

Ridanpaa M, Fodde R, Kielman M (2001) Dynamic expression and nuclear accumulation of β-catenin during the development of hair follicle-derived structures. Mech Dev 109:173–181

Rorive S, Eddafali B, Fernandez S, Decaestecker C, André S, Kaltner H, Kuwabara I, Liu F-T, Gabius H-J, Kiss R, Salmon I (2002) Changes in galectin-7 and cytokeratin-19 expression during the progression of malignancy in thyroid tumors: diagnostic and biological implications. Mod Pathol 15:1294–1301

Rotblat B, Niv H, André S, Kaltner H, Gabius H-J, Kloog Y (2004) Galectin-1(L11A) predicted from a computed galectin-1 farnesyl-binding pocket selectively inhibits Ras-GTP. Cancer Res 64:3112–3118

Rüdiger H, Gabius H-J (2001) Plant lectins. Glycoconj J 18:589–613

Sell S (2004) Stem cell origin of cancer and differentiation therapy. Crit Rev Oncol Hematol 51:1–28

Sharma UC, Pokharel S, van Brakel TJ, van Berlo JH, Cleutjens JPM, Schroen B, André S, Crijns HJGM, Gabius H-J, Maessen J, Pinto YM (2004) Galectin-3 marks activated macrophages in failure-prone hypertrophied hearts and contributes to cardiac dysfunction. Circulation 110:3121–3128

Shimura T, Takenaka Y, Fukumori T, Tsutsumi S, Okada K, Hogan V, Kikuchi A, Kuwano H, Raz A (2005) Implication of galectin-3 in Wnt signaling. Cancer Res 65:3535–3537

Siebert H-C, André S, Lu SY, Frank M, Kaltner H, van Kuik JA, Korchagina EY, Bovin NV, Tajkhorshid E, Kaptein R, Vliegenthart JFG, von der Lieth C-W, Jiménez-Barbero J, Kopitz J, Gabius H-J (2003) Unique conformer selection of human growth-regulatory lectin galectin-1 for ganglioside GM_1 versus bacterial toxins. Biochemistry 42:14762–14773

Silva WA Jr, Covas DT, Panepucci RA, Proto-Siqueira R, Siufi JL, Zanette DL, Santos AR, Zago MA (2003) The profile of gene expression of human marrow mesenchymal stem cells. Stem Cells 21:661–669

Solís D, Jiménez-Barbero J, Kaltner H, Romero A, Siebert H-C, von der Lieth C-W, Gabius H-J (2001) Towards defining the role of glycans as hardware in information storage and transfer: basic principles, experimental approaches and recent progress. Cells Tissues Organs 168:5–23

Sturm A, Lensch, M, André S, Kaltner H, Wiedenmann B, Rosewicz S, Dignass AU, Gabius H-J (2004) Human galectin-2: novel inducer of T cell apoptosis with distinct profile of caspase activation. J Immunol 173:3825–3837

Szolnoky G, Bata-Csorgo Z, Kenderessy AS, Kiss M, Pivarcsi A, Novak Z, Nagy Newman K, Michel G, Ruzicka T, Marodi L, Dobozy A, Kemeny L (2001) A mannose-binding receptor is expressed on human keratinocytes and mediates killing of Candida albicans. J Invest Dermatol 117:205–213

Takamura M, Sakamoto M, Ino Y, Shimamura T, Ichida T, Asakura H, Hirohashi S (2003) Expression of liver–intestine cadherin and its possible interaction with galectin-3 in ductal adenocarcinoma of the pancreas. Cancer Sci 94:425–430

Trombetta ES, Parodi AJ (2002) *N*-glycan processing and glycoprotein folding. In: Richards FM, Eisenberg DS, Kuriyan J (eds) Protein folding in the cell. Academic, San Diego, pp 303–344

Troy T-C, Turksen K (2005) Commitment of embryonic stem cells to an epidermal cell fate and differentiation. Dev Dyn 232:293–300

Ueda S, Kuwabara I, Liu F-T (2004) Suppression of tumor growth by galectin-7 gene transfer. Cancer Res 64:5672–5676

Villalobo A, Nogales-Gonzáles A, Gabius H-J (2005) A guide to signaling pathways connecting protein–glycan interaction with the emerging versatile effector functionality of mammalian lectins. Trends Glycosci Glycotechnol (in press)

Vyakarnam A, Lenneman AJ, Lakkides KM, Patterson RJ, Wang JL (1998) A comparative nuclear localization study of galectin-1 with other splicing components. Exp Cell Res 242:419–428

Walsh A, Chapman SJ (1991) Sugars protect desmosome and corneosome glycoproteins from proteolysis. Arch Dermatol Res 283:174–179

Watt FM (2002) The stem cell compartment in human interfollicular epidermis. J Dermatol Sci 28:173–180

Webb A, Li A, Kaur P (2004) Location and phenotype of human adult keratinocyte stem cells of the skin. Differentiation 72:387–395

Wollina U, Lange D, Paus R, Burchert M, Gabius H-J (2000) Expression of galectins-1 and -3 and of accessible binding sites during murine hair cycle. Histol Histopathol 15:85–94

Wu AM, Wu JH, Liu J-H, Singh T, André S, Kaltner H, Gabius H-J (2004) Effects of polyvalency of glycotopes and natural modifications of human blood group ABH/Lewis sugars at the Galβ1-terminated core saccharides on the binding of domain-I of recombinant tandem-repeat-type galectin-4 from rat gastrointestinal tract (G4-N). Biochimie 86:317–326

Yu Z, Weinberger PM, Provost E, Haffty BG, Sasaki C, Joe J, Camp RL, Rimm DL, Psyrri A (2005) β-Catenin functions mainly as an adhesion molecule in patients with squamous cell cancer of the head and neck. Clin Cancer Res 11:2471–2477